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Identification of key host proteins governing *Trichomonas vaginalis*: A host-parasite interaction study

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A thesis submitted in satisfaction of the requirements for the degree of

Master of Research

The School of Science and Health

Western Sydney University

2017

Supervisor: Dr Colin Stack

Acknowledgements

Firstly, I would like to thank my supervisor Dr Colin Stack for his guidance and support throughout the Master of Research degree. Through his support, he was able to guide me into becoming a true scientist and his expert knowledge in the field was incredibly valuable. I would like to thank Dr Sabine Piller for her expert advice and mentorship especially in learning many vital lab techniques including mammalian cell culturing. I would like to thank Dr Leah Stroud for her help and guidance both in and outside the lab, I am extremely appreciative for her help and patience while I learnt many experimental and data analytical techniques.

Secondly, I would like to thank my colleagues especially George Tsiotsioras, Iman Sohail, Hayden Rodwell, Lauren Rispen, Cassandra Thompson, Shayal Prasad, Anita Raposo and Caitlin McLuckie for their advice, mentorship and encouragement throughout the degree. These colleagues made surviving the masters journey much easier and worth it to be able to meet such wonderful people.

Lastly, I would like to thank my family, my Mum, my Dad, my brother and my girlfriend for their support throughout the degree, for their faith in me and for their endless advice which really helped me get through this journey.

Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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18/12/2017

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Abbreviations

ACN Acetonitrile

APAF Australian Proteome Analysis Facility

AP3B1 AP-3 complex subunit beta-1

ATCC American Type Culture Collection

BSA Bovine Serum Albumin

B7RC2 *Trichomonas vaginalis* strain

Cas9 CRISPR associated protein 9

CDC Centers for Disease Control

CP Cysteine Protease

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats

DMEM Dulbecco's Modified Eagles Medium

DMM Diamonds Modified Medium

DNA Deoxyribonucleic Acid

DTT Dithiothreitol

FC Fold Change

FDA Food and Drug Administration

FDR False Discovery Rate

FSC Forward Side Scatter

GC1qR Complement component 1Q subcomponent binding protein

GFP Green Fluorescent Protein

HCV Hepatitis C Virus

HIV Human Immunodeficiency Virus

HPLC High Performance Liquid Chromatography

HuMIF Human macrophage Migration Inhibitory Factor

HTRA2 Serine protease HTRA2

IDA Information dependent acquisition

IL Interleukin

Ig Immunoglobulin

LC-MS Liquid chromatography–Mass Spectrometry

LGALS1 Galectin-1

LPG Lipophosphoglycan

mESC mouse Embryonic Stem Cells

MOI Multiplicity of Infection

Naa10 N-alpha-acetyltransferase 10

PAM Protospacer Adjacent Motif

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PFO Pyruvate Ferredoxin Oxidoreductase

pH Potential of Hydrogen

PI Propidium Iodide

PTM Post Translational Modification

PWP1 Periodic tryptophan 1 homolog

RPS25 Ribosomal Protein S25

sgRNA single guide Ribonucleic Acid

siRNA small interfering Ribonucleic Acid

SWATH-MS Sequential Windowed Acquisition of All Theoretical fragment ion Mass Spectra

TBST Tris Buffered Saline Tween 20

TEAB Tetraethylammonium bromide

TGFβ Transforming Growth Factor Beta

TLR Toll Like Receptor

TOF-MS Time of Flight-Mass Spectrometry

TP53I3 Quinone oxidoreductase PIG3

TvLG Trichomonas vaginalis Lipoglycan

TvMIF *Trichomonas vaginalis* Homolog of macrophage Migration Inhibitory Factor

VK2/E6E7 Vaginal Epithelial cell line

VWA8 Von Willebrand factor A domain-containing protein 8

Abstract

Trichomonas vaginalis is a flagellated extracellular protozoan parasite that causes Trichomoniasis, a chronic infection in the vaginal and prostate epithelium of humans. Current treatments combating *T. vaginalis* infections are effective but there is a trend of increasing resistance to 5- nitromidazoles which are the main class of drugs used to treat trichomoniasis. To date, the majority of research has focused on the parasite and little research has involved the host. Parasites cannot live without their host which suggests that the host must be contributing something other than just a refuge for the parasite. This project studies *T. vaginalis* host–parasite interactions, specifically with the aim to identify how host proteins may be involved in the establishment and maintenance of *T. vaginalis* infection. The goal of this project was to interact *T. vaginalis* with HeLa mammalian cervical epithelial cells, with the view to identify upregulated and downregulated proteins using sequential window acquisition of all theoretical fragment ion spectra (SWATH) proteomics. A proof of principle study was also undertaken by knocking out the host receptor galectin-1 using CRISPR/Cas9 technology. Our hypothesis is that allowing interaction of *T. vaginalis* with HeLa cells there will be potentially a number of upregulated and downregulated host proteins that may be manipulated by *T. vaginalis*, which play a key role in the infection process.

22 proteins were found to be differentially expressed, including proteins that may be manipulated by *T. vaginalis*. Proteins that were identified were found to have many different biological functions including the regulation of apoptosis, inflammation, cell cycle regulation and attachment. These proteins may be studied in future experiments to identify their biological significance to *T. vaginalis* infection. The galectin-1

knockout study produced a partial knockout of galectin-1 and could be used in future attachment studies once further optimization of the CRISPR knockout is performed.

Chapter 1: Introduction

1.1 *Trichomonas vaginalis* overview: prevalence and risk to public health

Trichomonas vaginalis is a flagellated extracellular parasite that belongs to the phylum *Parabasalia*. As shown below in Figure 1, *T. vaginalis* is pear shaped and has a total of 5 flagella, one attached by an undulating membrane and 4 freely attached at the top of the parasite. *T. vaginalis* is a pathogenic protozoan resulting in a sexually transmitted infection of humans termed trichomoniasis (Kissinger 2015). This infection is estimated to infect 190 million people around the world per year (Van Der Pol 2007).

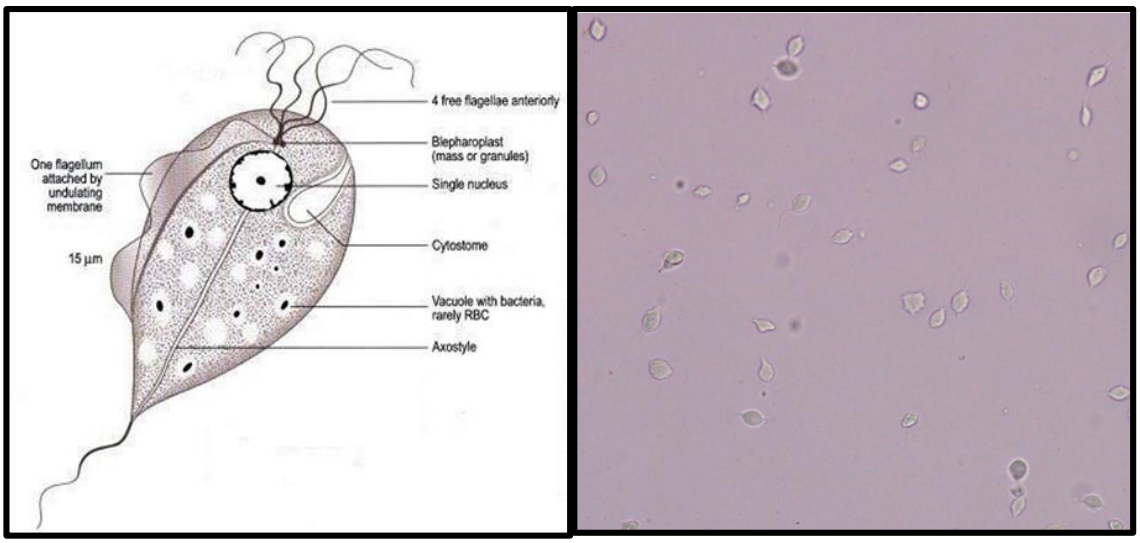

Figure 1. Structural Anatomy of *T. vaginalis* (Left) showing 4 free flagellae on the anterior side, a single flagellum attached by an undulating membrane, as well as a single nucleus (Microbe Online, 2016).20s image of free swimming B7RC2 strain *T. vaginalis* (Right) exhibiting the same shape and characteristics as the image on the left.

T. vaginalis' sole host is humans, where it resides in the urogenital tract of males and females dividing by binary fission (Kusdian & Gould 2014; Maritz et al. 2014). While the parasite colonizes both sexes, symptoms of infection occur more often in females than in males (Sutton et al. 2007). Trichomoniasis is the most common non-viral sexually transmitted infection causing inflammation of the vagina and/or the urethra

often accompanied with discomfort, pain and discharge (Mielczarek & Blaszkowska 2016).

Furthermore, *T. vaginalis* has also been implicated in a wide variety of health problems. Infection with *T. vaginalis* results in an increased risk of an individual acquiring HIV as well as issues associated with infertility and pre-term delivery such as low birth weights (Kissinger & Adamski 2013; Mielczarek & Blaszkowska 2016). Studies exploring the link between *T. vaginalis* and HIV have shown that the environment of the vagina plays an important role. The relationship between *T. vaginalis* and natural vaginal flora becomes imbalanced, increasing the procurement and transmission of HIV (Kissinger & Adamski 2013).

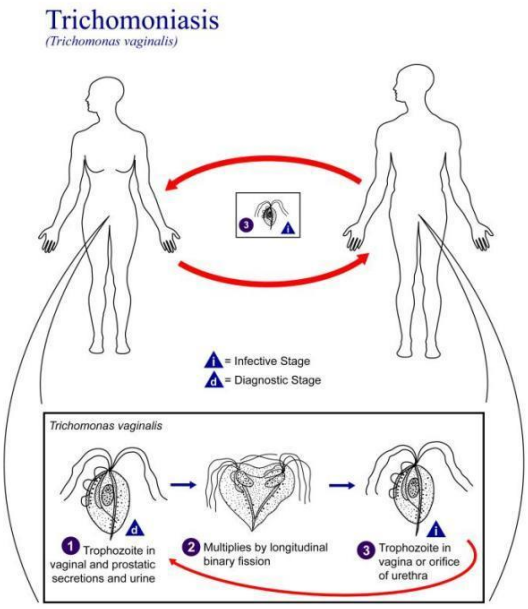

Figure 2. Life Cycle of *T. vaginalis* within the human host, the parasite multiplies by binary vision and is passed on by vaginal and prostatic secretions (CDC,2016).

Due to this parasites ability to attach to vaginal epithelial cells, *T. vaginalis* orchestrates a local immune response, leading to the inflammation of the vaginal epithelium in women and urethral epithelium in men. As shown in Figure 2, *T. vaginalis* is passed on by sexual contact passed on through vaginal and prostatic secretions. When the local immune response has begun, the inflammatory process disrupts the normal environment causing an imbalance of natural flora and altering the high pH of the environment to a lower pH. This causes an influx of immune cells into the area which potentially allows HIV to enter through lesions created by this response should a person have sexual contact with a HIV infected individual

(Kissinger & Adamski 2013). Furthermore, *T. vaginalis* causes hemorrhages in the mucosal membrane which increases the likelihood of HIV to be able to pass this protective barrier (McClelland et al. 2007).

The risk of developing cervical and prostate cancers is also increased, with one study showing a 3-fold increase of the detection of *T. vaginalis* in patients suffering from cervical cancer, although the mechanism behind this association is yet to be explored (Fowke et al. 2016; Sayed el-Ahl et al. 2002; Stark et al. 2009; Zhang, ZF et al. 1995). Studies considering the association of *T. vaginalis* and prostate cancer have linked chronic *T. vaginalis* infections with an increase in prostate cancer via *T. vaginalis* macrophage migration inhibitory factor (TvMIF). TvMIF acts similarly to its human counterpart, Human macrophage inhibitory factor (HuMIF) which has been shown to have a role in prostate cancer. TvMIF causes inflammation that triggers the proliferation of cells which promotes prostate cancer development (Twu et al. 2014). Currently the standard treatment for *T. vaginalis* infections is to treat with 5-nitroimidazoles such as the FDA approved metronidazole which is taken orally 500 mg twice a day for seven days or as a single 2g dose (Howe & Kissinger 2017). However, there has been a small increase at 5% in resistance to this drug leading to higher morbidity and relapsed infections which may mean there are host factors at play (Dunne et al. 2003; Kissinger 2015). This is further contended by the fact that infection by *T. vaginalis* is often underdiagnosed which is a big concern due to the important unfavourable health implications stated above (Javanbakht et al. 2013; Kissinger 2015; Wangnapi et al. 2015). Often when *T. vaginalis* is diagnosed and treated, re-infection will occur due to sexual contact with a partner who has not been treated for *T. vaginalis*. Due to this trend, prevention is extremely important as a person can immediately become re-infected (Alcaide et al. 2016; Hillier 2013; Kanno & Sobel

2003). Collectively, the increased prevalence of *T. vaginalis*, the trend of undiagnosed cases, the ease of reinfection and a small rise in metronidazole resistance poses great implications to public health around the world. More research must be performed on this parasite in order to improve public health outcomes in the future.

1.2 *T. vaginalis* pathogenicity: Molecular overview and host parasite interaction studies

In the study of parasitology, the focus of research efforts has been placed on the parasite while the host is often neglected. It is a possibility that by overlooking the contribution of the host to this interaction we are potentially restricting the amount of knowledge available. As the parasite requires the host to survive, it is imperative to understand what the host is contributing to the relationship. Perhaps by studying the host's contribution we will reveal a more holistic and clearer picture of the host and parasitic relationship and identify key areas that could lead to a potential cure. There have been *T. vaginalis* host – parasite interaction studies that have examined the role of parasite adhesion to host cells. These studies have focused on the roles of parasite surface proteins and molecules such as *T. vaginalis* lipoglycan (TvLG) which binds to host cell surface receptors (Ryan, de Miguel & Johnson 2011). Studies have also shown that when *T. vaginalis* interacts directly with host vaginal epithelial cells, they attach, shrink and change shape from their regular flagellated trophozoite state to a more rounded ameboid shape (Lin, WC et al. 2015; Petrin, Dino et al. 1998). Many studies have also explored *T. vaginalis* molecular mechanisms through host parasite interaction studies which have greatly increased our understanding of the parasite. Research has shown that *T. vaginalis* is able to manipulate its host environmental niche in order to initiate the inflammatory response which may help trick the immune system in increasing the parasites ability to survive within its host. *T. vaginalis* selectively

upregulates molecules such as proinflammatory mediators; cytokines, IL-8, IgA, IgG and Th1 that mediate the immune response (Fichorova 2009). *T. vaginalis* is able to suppress the immune response via adhesins such as AP65 and cysteine proteases (CP30) in order to induce apoptosis and suppress cytokines such as IL-10, TGFβ (Chang et al. 2004; Mundodi et al. 2004). *T. vaginalis* suppresses IL-10, TGFβ, monocytes and macrophages in order to avoid detection and destruction (Chang et al. 2004; Fichorova 2009). Combined, it seems that *T. vaginalis* selectively induces and suppresses immune responses to manipulate the immune system in to destroying host cells, which aids *T. vaginalis* in gaining nutrients from the microenvironment while also avoiding destruction via cytokines.

Furthermore, *T. vaginalis* is able to phagocytose epithelial cells and bacteria in order to gain the essential nutrients it needs to survive and replicate (Petrin, Dino et al. 1998). *T. vaginalis* actively releases an array of secretory proteins, some that aid *T. vaginalis* in adhering (adhesins) and others to break down proteins (proteases). These secreted proteins include lipophosphoglycan (LPG), adhesins, cysteine proteases and cytoskeletal proteins (Hernández, Marcet & Sarracent 2014). *T. vaginalis* LPG directly interacts with host galectin–1, a surface receptor located on the surface of epithelial cells, triggering interleukin 8 expression and facilitating parasite adhesion (Lin, W-C et al. 2015).

Through the use of adhesins, *T. vaginalis* anchors itself to host epithelial cells and to facilitate the uptake of iron in the environment. This is needed in order to facilitate growth as *T. vaginalis* requires iron as a key component in its metabolism, for example as a cofactor in enzymes and regulatory proteins (Beltrán et al. 2013; Lehker & Alderete 1992). *T. vaginalis* has been seen to regulate genes when environmental iron is limited. It responds to such environments by upregulating hydrogesomal iron sulfur

cluster proteins while downregulating enzymes used in carbohydrate metabolism such as Fe-Fe hydrogenase and pyruvate ferredoxin oxidoreductase (PFO) (Beltrán et al. 2013). This shows that *T. vaginalis* is able to differentially express genes in direct response to their environment increasing its survivability for example, in the vaginal region where iron availability constantly changes. Other proteins such as adhesin proteins AP23, AP33, AP51 and AP65 have been found to be synthesized by *T. vaginalis* enabling the parasite to acquire iron from its host (Garcia et al. 2003). Surface AP65 expressed from *T. vaginalis* is able to adhere to erythrocytes enabling iron to be taken into the parasite (Vieira et al. 2015). Increased expression of *T. vaginalis* surface adhesins was demonstrated to occur following contact with vaginal epithelial cells which suggests a contact dependent mechanism for adherence at play (Garcia et al. 2003).

Several studies have explored this host–parasite interaction through co-culture methods and have discovered that *T. vaginalis* is capable of altering gene expression in host cells. Specifically, those involved in the immune response resulting in differential expression of host cytokines IL-1α and IL-1β (Kucknoor, Mundodi & Alderete 2005). IL-1β mediates inflammatory responses and increases tight junction permeability (Lin, W-C et al. 2015). Tight junctions aid in the communication between adjacent cells and the disruption of these junctions can affect macromolecule trafficking and trigger an inflammatory response which allows *T. vaginalis* to uptake the nutrients it needs to survive and proliferate (Lee 2015).

While *T. vaginalis* employs contact dependent mechanisms for attachment, nutrient uptake, survival and proliferation, *T. vaginalis* also utilizes contact independent mechanisms concurrently. Contact independent mechanisms include the secretion of enzymes such as cysteine proteases. Cysteine proteases (CPs) are an important class

of enzymes secreted and utilized by *T. vaginalis* to break down host proteins and assists in parasite adherence. These enzymes have been implicated in parasite cytotoxicity, adherence, metabolism, haemolysis and evasion of the immune response (Hernández, Marcet & Sarracent 2014). One study by Mendoza- Lopez et al (2000) showed that cysteine proteases such as CP30, a key virulence factor, is used by the parasite in order to bind to the surface of mammalian cervical epithelial cells, but is only partly responsible for parasite attachment (Mendoza-Lopez et al. 2000). Cysteine proteases can counter host immune responses by degrading IgG and IgA, dampening the humoral immune response further aiding parasite survival (Provenzano & Alderete 1995). Through host–parasite interaction studies using the cysteine protease inhibitor E-64, cysteine proteases have been identified as *T. vaginalis* virulence factors, such virulence factors including CP4, CP12, CP39 (Figueroa-Angulo et al. 2012).

The genome of *T. vaginalis* was published in 2007 which has been an extremely useful tool in understanding the underlying molecular mechanisms of this parasite (Carlton, J.M. et al. 2007). The genome contains 446 proteases, of which 220 are cysteine proteases (Carlton, J. M. et al. 2007). *T. vaginalis* has one of the largest genomes of a protist sequenced. The genome is 160 Mb and has been suggested to encode for around 46,000 unique proteins (Smith & Johnson 2011). The genome also provided insights into the evolutionary pathways of *T. vaginalis*. *T. vaginalis* was found to have expanded its genome size significantly, especially the parasites membrane trafficking machinery. This expansion or duplication of genes is used to secrete proteins that have capabilities including the ability to endocytose host proteins, phagocytose host cells as well as bacteria. This has improved *T. vaginalis'* ability to adapt to its host's environment (Carlton, Jane M. et al. 2007). The genome of *T. vaginalis* also consists of 152 genes that have been inherited through lateral gene transfer from prokaryotes,

which may have also aided *T. vaginalis* adaption to its environment which it shares with several prokaryote species (Carlton, Jane M. et al. 2007). One such gene that may have been acquired through lateral gene transfer encodes for the enzyme N-acetylneuraminate lyase. This enzyme metabolises sialic acids which has been identified to be similar in different organisms and allows these organisms to acquire sialic acid from its host environment (Singh, S et al. 2012).

Studies have considered the effects of host environmental factors on the virulence of *T. vaginalis* and have identified several factors that modulate the expression of genes in the parasite (Figueroa-Angulo et al. 2012). Regulation by iron was identified as an important contributor to differential gene expression, which is essential for growth and metabolism. Given that *T. vaginalis* survives on the cervical epithelium, *T. vaginalis* must adapt to an environment that is constantly changing due to the menstrual cycle. Interestingly, trichomoniasis symptoms have been observed to be more severe during the menstrual cycle where there is a higher pH and the menstrual blood supplies a rich amount of iron for the parasite to utilize (Cudmore et al. 2004; Petrin, D. et al. 1998).

1.3 Role of galectin–1 in *T. vaginalis* adhesion to mammalian cells

Galectin–1 is a host β-galactoside binding protein that binds lectins, which is encoded by the gene *LGALS1*. Galectin-1 is found on the cell surface of human epithelial cells and is part of a larger family of galectins which have diverse biological roles (Liu & Rabinovich 2010). Galectins are able to mediate interactions between cells and can also cross link to each other in order to facilitate adhesion through the binding to glycans on neighboring cells (Zick et al. 2004). Galectins have been implicated in many diseases including HIV, cancer and chronic inflammation (Curciarello et al. 2014; Zhang, P et al. 2014). Studies have suggested a role for host galectin-1 and

galectin-3 in *T. vaginalis* pathogenicity, acting as pathogen recognition receptors to *T. vaginalis* LPG (Fichorova, R. N. et al. 2016). Host galectin–1 and 3 were silenced via siRNA in a host-parasite interaction study which found that silencing galectin-1 suppressed chemokine responses to *T. vaginalis*. On the other hand, silencing galectin-3 reduced immune responses, such as IL-8 responses to *T. vaginalis* LPG (Fichorova, R. N. et al. 2016). *T. vaginalis* could reduce extracellular levels of galectin-3 as well as bind to galectin-1 to reduce chemokine expression (Fichorova, R. N. et al. 2016).

Due to the morphology of *T. vaginalis*, LPG covers the surface of this parasite which contains a large amount of galactose. This galactose can be recognized and bound by host cell galectins and may possibly facilitate the establishment of infection. *T. vaginalis* LPG is able to attach to host cell galectin through cross linking of the glycan to host galectin (Singh, BN et al. 2009). This implies that host surface galectin-1 plays a vital role in parasite adherence (Okumura, Baum & Johnson 2008). The same study that silenced host cell galectin -1 using siRNAs found that reducing the expression of galectin-1 negatively impacted the ability for *T. vaginalis* to bind to host epithelial cells (Okumura, Baum & Johnson 2008; Vasta et al. 2012).

When combining this information about the role of galectins, in particular galectin-1, we can see that there are potentially multiple roles of galectin that *T. vaginalis* exploits in order to survive and proliferate in its niche environment. These roles include parasite attachment and evasion of the immune system through suppressing chemokines and IL-8 responses. This suggests that galectins such as galectin-1 should be considered to be prime targets for further study to determine how important host galectins are and if they could potentially act as novel targets for therapies in the future.

1.4 CRISPR gene knockout study

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9) is a gene editing system derived from the natural defence systems of bacteria such as *Streptococcus pyogenes* which can be used to induce a mutation in target DNA thereby knocking the corresponding gene out (Munoz et al. 2014). CRISPR is a relatively new technology which was discovered and developed by multiple scientists around the world since 1993 but it took until 2013 to be used as a genome editing technique (Lander 2016). As this technology is still relatively young, the potential impact of this extremely precise and cost-efficient technology is still being debated, especially in the context of the ethics of human genome editing (Go & Stottmann 2016). The hope is that CRISPR could be used in the future to treat numerous diseases, especially genetic diseases such as cystic fibrosis, down syndrome and sickle cell anemia (Go & Stottmann 2016).

Currently CRISPR/cas9 systems have been mainly used on mammalian and plant genomes, with some studies expanding into eukaryotes such as *Toxoplasma gondii* in order to gain further understanding on the function of specific genes within the parasites genome (Sidik et al. 2014). The ability to genetically engineer parasites such as *T. gondii* using CRISPR/ cas9 is still quite new and yet CRISPR has been shown to be an efficient tool for genome editing (Sidik et al. 2014). CRISPR has also been successfully used in *Plasmodium falciparum* and *Leishmania donovani* providing functional analysis of genomes, improving and increasing the efficiency of research into the function of genes in these parasites (Ghorbal et al. 2014; Zhang, WW & Matlashewski 2015). Currently there have been no published studies using CRISPR in *T. vaginalis*, which would be imperative in further understanding the function and

discovery of key genes in this parasite. Despite CRISPR/cas9 gene editing has not been utilised in *T. vaginalis*, CRISPR gene editing may potentially be used to identify functional relations between *T. vaginalis* and host cells during interaction studies. By editing genes in host mammalian epithelial cells, novel insights into the function and roles of proteins such as galectin-1 in mammalian cells may be discovered. In this thesis, galectin-1 was attempted to be knocked out as a proof of concept study, to further analyse the role of host surface galectin-1 on the adherence of *T. vaginalis* in future studies.

Although there are various CRISPR/Cas9 systems, the system that will be used in this study contains dual nickases that cause breaks in both strands of DNA at specific target locations. CRISPR uses single guide RNA to target a specific sequence as well as a scaffold domain in order to bind Cas9 (DNA endonuclease) (Munoz et al. 2014). The guide RNA is complementary to the bases of a specific sequence of DNA of the gene that is being targeted for knockout or knockdown, thus enabling the sgRNA to target a specific region in the genome. The target DNA must be directly adjacent to a protospacer adjacent motif (PAM) in order for Cas9 to successfully bind and cleave the DNA (Ran et al. 2013). Once bound to this DNA sequence the scaffold DNA allows Cas9 to specifically target this region of DNA and make cuts, which then induces DNA repair mechanisms. Through homologous repair, the target DNA mutates leading to the knockdown of the target gene of interest (Munoz et al. 2014). Due to the chance of off target mutations, dual Cas9 nickases will be used to nick target DNA on both strands. As shown in Figure 3, dual nickases target two specific sites which increases the specificity of Cas9 endonuclease activity and reduces the chances of off-target mutations occurring (Ran et al. 2013). Figure 4 shows the components of the double nickase plasmid supplied by Santa Cruz Biotechnologies (2017). One

plasmid in the pair contains a puromycin resistance gene which was used in order to select for transfected cells.

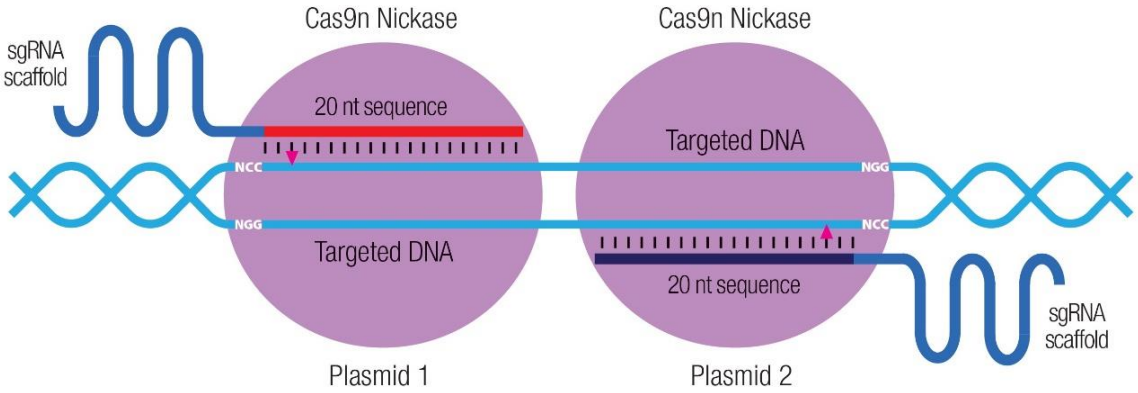

Figure 3. CRISPR double nickase diagram showing two target sites of the nickase plasmids. The dual nickases target complementary strands of DNA opposite of the targeted DNA in order to induce a mutation causing a knockout/down (Santa Cruz, 2017).

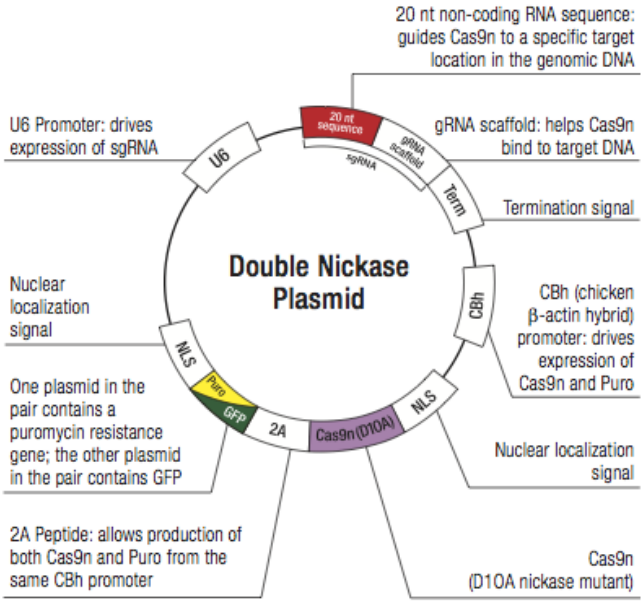

Figure 4. Components of a double nickase plasmid, showing the inclusion of a puromycin resistance gene to be used for selection as well as a GFP gene that can be used for cell sorting (Santa Cruz, 2017).

There have been a few studies knocking out genes in the host of *T. vaginalis,* while most have used methods such as siRNA to knockdown the genes. A study considering the effects of galectin-1 and galectin-3 expression on *T. vaginalis* adherence used siRNA to knockdown galectin-1 and 3 expression within host epithelial cells. They found that reducing galectin-1 and 3 expression correlated to a partial reduction in attachment to the host, suggesting that there are multiple factors that contribute to *T. vaginalis* ability to attach to its host (Fichorova, Raina N. et al. 2016). The same researchers found that *T. vaginalis* is able to regulate the availability of galectin in order to suppress chemokines and aid the parasites survival (Fichorova, Raina N. et al. 2016).

1.5 SWATH-MS proteomic study

Proteins are vital to the function of all living organisms. The global study of proteins, proteomics, is a vital technique used to understand the role(s) of proteins within an organism. Proteomics is a large-scale study of all proteins in an organism at a given point in time, that can provide insights into what proteins are expressed in response to a particular stimulus. This data can then be compared to genomic data in order to identify the correlated genes. Proteins are the final product of genes which highlights why understanding the functions and structure of proteins including post translational modifications (PTMs) can provide insights into the phenotype and functional characteristics of an organism (Graves & Haystead 2002). Proteomics has been a useful tool for many studies including the study of human parasites including *T. vaginalis*. For example, proteomics has been used to identify the abundance of cysteine proteases and has contributed to their identification as virulence factors that enzymatically break down host proteins (Sommer et al. 2005).

There are numerous methodologies utilized in protein analysis ranging from 2 dimensional (2D) gels to large scale shot gun proteomics that uses mass spectrometry in tandem with liquid chromatography columns to separate and identify proteins. There are positives and negatives for all methodologies. For example, 2D gels are able to separate individual proteins from complex samples, but require a lengthy manual process leading them to be more prone to human error (Bunai & Yamane 2005).

The methodology used in this study was sequential window acquisition of all theoretical mass spectra (SWATH) a type of shotgun proteomics performed at the Australian Proteome Analysis Facility (APAF) at Macquarie University. SWATH is a label free mass spectrometry based proteomic tool that is able to detect a large amount of proteins (> 6000) from the lysates of whole cells (Huang et al. 2015). As shown in Figure 5, SWATH uses data independent acquisition in combination with a peptide spectral library before statistical analysis is performed. SWATH can be used to accurately identify proteins across a wide range of samples, while also quantifying the relative abundance of the proteins (Huang et al. 2015).

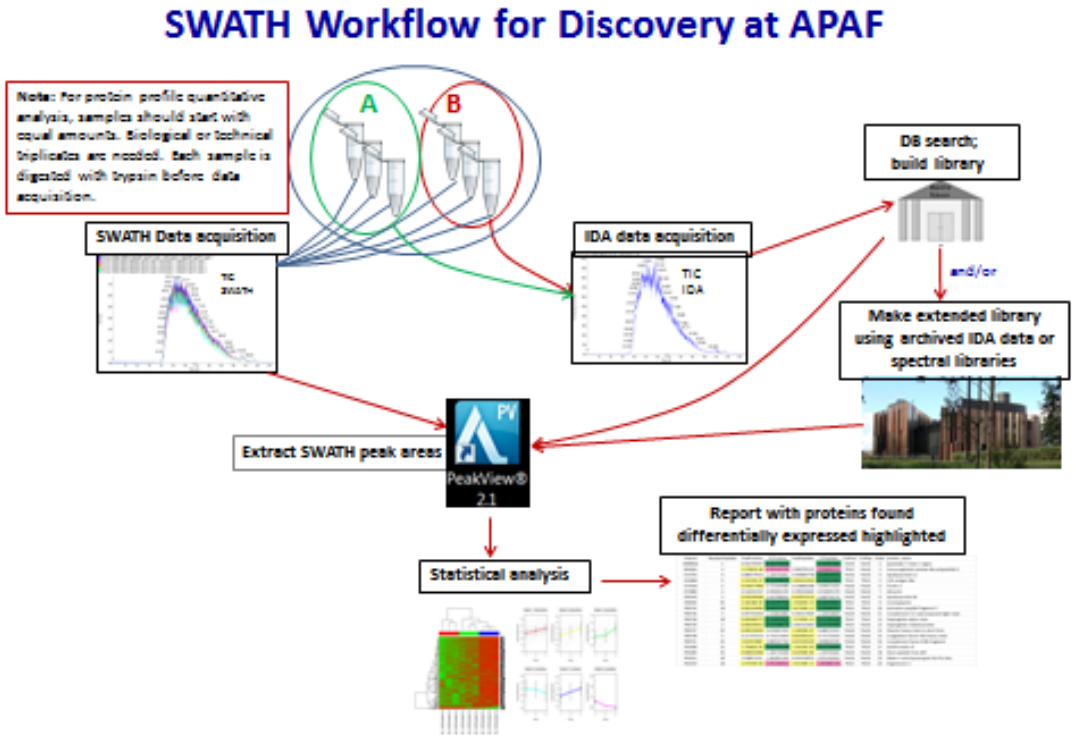

Figure 5. SWATH proteomic workflow at the Australian analysis facility, samples are digested with trypsin to break up the proteins into fragments before run through MS, a database is searched using the independent data generated by MS, the data is statistically analyzed and differentially expressed proteins with a *p* value of < 0.05 are highlighted (APAF, 2010).

SWATH analysis was performed on mammalian HeLa cells following direct interaction with *T. vaginalis* for a defined amount of time. This methodology aimed to potentially identify proteins that have become upregulated or downregulated in the host cells following *T. vaginalis* attachment providing a proteome wide scale analysis. This potentially generated a knowledge of key proteins and/or pathways used by *T. vaginalis* that could be used in future studies and to develop novel therapies in the future.

Chapter 2: Aims

2.1 Aims

There are two specific aims of this study Aim 1: is to investigate changes in protein expression in host mammalian cervical epithelial cells following direct interaction of *T. vaginalis* parasites and HeLa cells. Aim 2: a proof of concept study to successfully knockout host galectin-1 using CRISPR/ Cas9 technology. Combined, this study will reveal new insights into host–parasite protein–protein interactions gaining further understanding of host–parasite interactions.

Chapter 3: Methods

3.1 Culturing methods

3.1.1 Mammalian cell culturing

HeLa cells (ATCC® CCL-2™) were cultured *in vitro* at 37°C, 5% CO_2 in Dulbeccos Modified Eagle's Medium (DMEM) (D5546-500ML, Sigma-Aldrich) supplemented with 10% foetal bovine serum (SFBS-F Bovogen) and a final concentration of 10 U/mL penicillin and 10 µg/mL (P4333 Sigma-Aldrich) streptomycin antibiotics. HeLa cells were passaged at 80% confluency by removing media and washing in pre-warmed (37°C) phosphate buffered saline (PBS) (D8537-500ML Sigma-Aldrich). 1 mL of Trypsin-EDTA (0.25%, 0.02%) was added and the cells were placed in a 35°C incubator for 5 minutes. Trypsinised cells were placed in a 15 mL tube and spun down in a centrifuge at 300 g for 5 minutes. The pellet was washed in fresh DMEM before being centrifuged at 300 g and was repeated for an additional 5 minutes. HeLa cells were resuspended in fresh DMEM and an aliquot was taken in order to count viability using a haemocytometer. 20 µL of cell suspension was mixed with 20 µL of trypan blue in a 1:1 ratio, stained and unstained cells were counted in order to count total number of viable cells using the formula live cells/ total cells x 100. Cells > 95% viability were split at a ratio of 500 µL cells to 4.5 mL DMEM and aliquoted into sterile T-25 flasks.

3.1.2 Parasite culturing

T. vaginalis strain B7RC2 (ATCC® 50167™) was cultured in Diamonds modified medium containing tryptose (211713 BD Biosciences), yeast extract (212750 BD Biosciences), maltose (M5895 Sigma-Aldrich), cysteine (C7477 Sigma-Aldrich), ascorbic acid (A4544 Sigma-Aldrich), KH_2PO_4 (0781 Amresco), K_2HPO_4 (P3786 Sigma-Aldrich) and pH was adjusted to 6.2 using HCl (302331 Sigma-Aldrich). Media

was autoclaved before 10 µg/ ml of iron solution (203505-25G, Sigma-Aldrich) consisting of 0.1 g/mL $Fe(NH_4)2(SO_4)6H_20$ and 0.01 g/mL 5–sulfosalicylic acid (0610-250 Amresco) was filter sterilized and added to the media. Media was supplemented with 10% heat inactivated horse serum and 1x Penicillin- Streptomycin cocktail (P4333-100ML Sigma-Aldrich) prior to parasite incubation. *T. vaginalis* was grown in an axenic environment in an incubator set at 37°C plus 5% CO_2 and passaged every 2 days. Parasite numbers and viability were counted using a haemocytometer and parasites were stained in propidium iodide (P4170-10MG, Sigma-Aldrich) to check viability of the cells. 1 µL of propidium iodide (1ug/ml) was mixed with 999 µL of parasites and mixed. 10 µL of propidium iodide/ cell mix was pipetted onto a haemocytometer and then placed under a fluorescent microscope. Propidium iodide is excluded from live cells and is excited at 535 and has an emission at 617 nm. Live and dead cells (stained) are counted to acquire a percentage by the formula: Viable cells divided by total cells x 100. All parasites used in each experiment were >95% viable. Parasites were not subcultured for more than 6 subcultures before a fresh stock was revived in order to maintain the parasites virulence.

Stocks of *T. vaginalis* were cryopreserved as per ATCC guidelines within the first two subcultures of a revived stock to keep the passage number of parasites as low as possible (ATCC, 2017).

3.2 Host- Parasite interaction study methods

3.2.1 SWATH-MS Host-parasite interaction

HeLa cells were grown to 80% confluency in 6 T-25 flasks while B7RC2 strain *T. vaginalis* parasites were cultured as described above and counts were taken using a haemocytometer to obtain a concentration of 1 x 10^6 parasites/ml and > 95% viability in a 50 mL Eppendorf tube. Parasites were spun down at 800 g for 5 minutes and adjusted to 1 x 10^6 parasites by resuspending in 1:1 DMM/DMEM. Confluent HeLa cells were washed twice in sterile mammalian culture grade phosphate buffered saline and 10 mL of pre-warmed 1:1 DMM/DMEM media mixture was added to the T-25 flasks. At the start of the interaction 5 x 10^4 parasites/mL (MOI 1:5) were added to 3 flasks while 3 control flasks were given extra media equal to the volume of parasites to keep the volume of media in the flasks consistent (250 µL equivalent to the amount added to the treated flasks equal to 5 x 10^4 parasites/mL). The flasks were placed in an incubator at 37°C plus 5% CO_2 for 30 minutes. After 30 minutes, the interaction was broken by placing the flasks (including controls) on ice for 10 minutes. The media was removed and the cells were washed twice with 5 mL of ice-cold PBS. Between each wash, the flasks were lightly hit on the sides (Using hands) in order to aid the dislodging of the parasites. Flasks were inspected visually using a microscope between each wash to detect if the parasites were washed off completely. The HeLa cells were immediately lysed in 200 µL of ice cold lysis buffer (1% SDC (D750 Sigma-Aldrich), 0.1 M Triethylammonium bicarbonate (T7408 Sigma-Aldrich), 150 mM NaCl (s9888 Sigma-Aldrich)) + 1x protease inhibitor cocktail (P8340 Sigma-Aldrich) + 10 mM NaF (201154 Sigma-Aldrich). HeLa cells were lysed for 1 hour on ice on a shaker before the cells were scraped off each flask using a new pre-chilled sterile cell scraper for each individual flask. The lysate was transferred to pre-chilled 1.5 mL Eppendorf

tubes and spun down to remove bubbles at 10,000 g for 5 minutes at 4°C. The samples were then sonicated on ice at 65 ampules 5 seconds on, 15 seconds off and repeated for a total sonication time of 35 seconds using a Unisonics fx8 sonicator. The samples were then spun down again at 10,000 g for 5 minutes at 4°C and the lysate was transferred to new pre-chilled 1.5 mL Eppendorf tubes to remove any cellular debris. A BSA protein concentration assay (Bio-Rad detergent compatible) (Bio-Rad DC Protein Assay Bio-Rad) used to quantify the total amount of proteins in each sample. The protein concentration assay measured triplicates of the samples against seven known concentrations of bovine serum albumin: 0, 0.25, 0.5 ,1 ,2 ,3 ,4 mg/ mL in triplicate on a 96 well plate. 25 µL of reagent A was pipetted into each well before 5 µL of each standard and sample (triplicates) were added. 200 µL of reagent B was added to make up a total of 230 µL in each well. The plate was placed in darkness for 15 minutes to allow for the colimetric reaction to occur. The plate was read on a spectrophotometer plate reader (ThermoScientific Multiskan® EX) at an absorbance of 595 nm. Data collected was analysed using Excel to create a standard curve from the standard readings and the Control and treated samples were compared to these standards to obtain the relative concentrations of the samples.

3.2.2 SWATH mass spectrometry

SWATH mass spectrometry was performed at the Australian Proteome Analysis Facility at Macquarie University. **The following methods were sent from APAF with a report outlining the steps from sample preparation to quantitation (Pages 33-37).**

3.2.3 Sample Preparation

50 ug of protein from each sample was diluted using 100 mM TEAB buffer containing 1% sodium deoxycholate giving a final concentration of 50 µL. Samples were reduced

with dithiothreitol (final concentration of 10 mM DTT) for 30 minutes at 56°C followed by alkylation with iodoacetamide (final concentration of 25 mM IAA) at room temperature for 30 minutes. Samples were digested with 1 µg of trypsin (1 µg trypsin per 50 µg of protein) for approximately 16 hours at 37°C. Formic acid was added to make a final concentration of 1% to precipitate sodium deoxycholate. Samples were centrifuged at 10,000g for 10 min and supernatant containing peptides was collected in a fresh tube and dried using vacuum centrifugation.

Prior to LC-MS analysis, samples were reconstituted using 25 µL of 2% acetonitrile, 97.9% water and 0.1% formic acid. Sonicated and vortexed briefly followed by high speed centrifugation for 10 minutes. Supernatant containing peptides were subjected to LC-MS/MS analysis.

3.2.4 High-reverse-phase fractionation

A pool of peptides was prepared using approximately 23 µg of each sample (total ~200 µg) and cleaned up with C18 Sep Pack Light Cartridges (Waters, Millford MA) and dried down using a speed-vac. Samples were reconstituted with 5 mM ammonia solution (pH 10.5) and loaded onto an Agilent 300 Extend C18 column (2.1 mm x 150 mm, 3.5 µm, 300Å). Using a 1260 quaternary HPLC system, peptides were separated using a linear gradient of 5 mM ammonia solution with 90% acetonitrile (pH 10.5) starting from 3% to 30% for 55 minutes, and then to 70% for 10 minutes and finally to 90% for another 5 minutes at a flow rate of 300 µL/min. Peptides were separated into a total of 90 fractions that were consolidated into 13 for liquid chromatography tandem mass spectrometry (IDA-LC–MS/MS) analysis.

3.2.5 1D Information dependent acquisition (IDA)

HpH fractionated peptides were subjected to 1D-IDA nanoLC MS/MS analysis (IDA-LC–MS/MS). Each sample was injected onto a reverse-phase trap for preconcentration and desalted with 2% acetonitrile, 97.9% water and 0.1% formic acid, at 5 µL/min for 3 minutes. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using linear solvent gradients of 5-35% of mobile phase B over 120 min at a flow rate of 600 nL/min. After peptide elution, the column was cleaned with 95% solvent B for 6 minutes and then equilibrated with 95% mobile phase A for 10 minutes before next sample injection. The reverse phase nano-LC eluent was subject to positive ion nano-flow electrospray analysis in an information dependent acquisition mode (IDA).

In the IDA mode, a TOF-MS survey scan was acquired (m/z 350-1500, 0.25 second), with the 20 most intense multiply charged ions (2+ to 4+; exceeding 200 counts per second) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 100 milliseconds in the mass range m/z 100–1800 with rolling collision energy.

3.2.6 Data independent acquisition (SWATH)

Each sample (2 µg) was injected onto a peptide trap for pre-concentration and desalted with 0.1% formic acid, 2% ACN, at 5 µL/min for 3 minutes. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using linear solvent gradients of 5-35% of mobile phase B over 60 min at a flow rate of 600 nL/min. After peptide elution, the column was cleaned with 95% mobile phase B for 6 minutes and then equilibrated with 95% mobile phase A for 10 minutes before next sample injection. The reverse phase nano-LC eluent was subject to positive ion nanoflow electrospray analysis in a data independent acquisition mode (SWATH).

For SWATH MS, m/z window sizes were determined based on precursor m/z frequencies (m/z 400–1250) in previous IDA data (SWATH variable window acquisition, 100 windows in total). In SWATH mode, first a TOF-MS survey scan was acquired (m/z 350-1500, 0.05 sec) then the 100-predefined m/z ranges were sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 30 milliseconds in the mass range m/z 350-1500 with rolling collision energy optimised for lowed m/z in m/z window +10%. To minimize instrument condition caused bias, SWATH data were acquired in random order for the samples with one blank run between every sample injection.

3.2.7 IDA data analysis

The 13 data files generated by IDA-MS analysis of HpH fractioned samples were searched with ProteinPilot (v5.0) (Sciex) using the ParagonTM algorithm in thorough mode. Uniprot (Uniprot_Human_review_Sept_2017, downloaded from http://www.uniprot.org/proteomes/UP000005640) database containing 42,202 human proteins was used for searching the data. Carbamidomethylation of Cys residues was selected as a fixed modification. An unused Score cut-off was set to 1.3 (95% confidence for identification). Resultant consolidated data file was used as ion-library with further modifications as described in the following section.

3.2.8 SWATH quantitation

SWATH data files and ion-library were imported into PeakView (v2.1) software and retention times were aligned using six high abundant endogenous peptides. Following the retention time alignment, resultant ion library was manually modified to include peptides with following most frequently observed modifications; cabamidomethyl (C, @N-term), Oxidation (M), dioxidation (W), acetylation proteins N-term, Gln->pyro-Glu and Glu->pyro-Glu. SWATH data were extracted using PeakView (v2.1) with the

following parameters: Top 6 most intense fragments of each peptide were extracted from the SWATH data sets (75 ppm mass tolerance, 10 min retention time window). Modified peptides (as mentioned above) were included and shared peptides were excluded. After data processing, peptides (max 100 peptides per protein) with confidence ≥ 99% and FDR ≤ 1% (based on chromatographic feature after fragment extraction) were used for quantitation.

The extracted SWATH protein peak areas were analysed using APAF in-house statistical analysis program. The protein peaks areas were log transformed, normalised to the total protein peak area for each run and subjected to one sample t-test to compare relative protein peak area between the sample groups (Control vs B7RC2). T-test p-value smaller than 0.05 and fold change ±1.5 were highlighted as differentially expressed proteins.

3.3 Galectin-1 Knockout methods

3.3.1 Puromycin selection

Puromycin concentration was optimized prior to CRISPR knockout of HeLa cells by growing HeLa cells in a 6 well plate to 80% confluency in antibiotic free DMEM + 10% FBS. Puromycin was added to each well of the 6 well plate in a range of concentrations from 0 µg, 1 µg, 2.5 µg, 5 µg, 7.5 µg and 10 µg/ mL. The plate was left to incubate for 5 days and cells were observed every 24 hours. Every 24 hours cells were observed visually for signs of death (Cells detaching, rounded cells) and a % of live cells were recorded for each well per day. The plate was washed every 48 hours and fresh puromycin was added to reduce the number of dead cells in each plate. The lowest concentration to kill 100% of the cells in 3 days (2.5 µg) was used in the CRISPR knockout experiment.

3.3.2 CRISPR Knockout

CRISPR knockout of HeLa cells was performed in 6 well plates. Confluent HeLa cells grown in a T-25 flask were washed in PBS and trypsinised in 0.25% Trypsin 0.02% EDTA for 5 minutes at 35°C. Cells were re-suspended in DMEM supplemented with 10% foetal bovine serum and a 1:1 cell:trypan blue aliquot was placed on a heamocytometer to check viability and cell numbers. 1.5 x 10^5 Cells with > 95% viability were aliquoted into 3 mL of DMEM and grown for 24 hours. Transfection was performed when the cells reached between 40-80% confluency. Cells were washed with PBS and 3 mL of antibiotic free DMEM + 10% FBS was added to the cells prior to the start of transfection. 1 µg (25 µL) of galectin-1 plasmid DNA (sc-400941-NIC, Santa Cruz) was added dropwise to plasmid transfection medium (sc-108062, Santa Cruz) into a final volume of 150 µL (Reagent A) and left to stand at room temperature for 5 minutes. Separately, 5 µL of UltraCruz transfection reagent (sc-395739, Santa Cruz) was added dropwise to 145 µL of plasmid transfection medium to a final volume of 150 µL (Reagent B) and left to stand at room temperature for 5 minutes. Both plasmid DNA and transfection reagent (Reagents A and B) solutions were combined (Total 300 µL) after 5 minutes of incubation and vortexed before incubating for an additional 20 minutes at room temperature. The total solution (300 µL) was then added dropwise to a single well of the 6 well plate and mixed by swirling gently. This was repeated 3 times for triplicates, alongside the treatment, untreated/ control HeLa cells were grown in the other 3 wells. The 6 well plate was incubated at 37°C + 5% CO2 for 24 hours. Media Was then replaced with fresh DMEM supplemented with 10% foetal bovine serum (no antibiotics) to remove dead cells and then incubated for an additional 24 hours. Cells were then selected using 2.5 µg of puromycin for 48 hours until all cells were dead in the 3 control wells. Cells were then

washed and left to grow up to confluency before being split into two T-25 flasks prior to Western blot analysis to quantify the effectiveness of the knockout.

3.3.3 Western blot analysis of knockout

Western blot analysis of galectin-1 expression was performed on CRISPR gene edited HeLa cells as well as control HeLa cells. Confluent HeLa cells were washed twice in PBS to remove foetal calf serum and lysed in 200 µL ice cold lysis buffer (1 % SDC, 0.1 M Triethylammonium bicarbonate, 150 mM NaCl) + 1x Protease Inhibitor cocktail (final volume) (P8340 sigma) and left on ice for 1 hour. Lysed cells were scraped with a cell scraper for 5 minutes and the accumulated lysate was transferred into a pre-chilled 1.5 mL Eppendorf tube. The lysate was spun down at 10,000g for 5 minutes at 4°C to remove bubbles. The lysed samples were then sonicated on ice at 65 ampules 5 seconds on, 15 seconds off and repeated for a total sonication time of 35 seconds. The samples were then spun down at 10,000g for 10 minutes at 4°C and the lysate was transferred to new pre-chilled 1.5 mL Eppendorf tubes to remove cellular debris. An aliquot from the samples was taken to perform a BSA concentration assay before the samples were stored at -80°C. 30 µg of protein from each sample was denatured and reduced in 4x Laemmli sample buffer (161-0747 Bio-Rad) supplemented with 10% 2-Mercaptoethanol (161-0710 Bio-Rad) at a ratio of 3:1 by boiling at 95°C for 7 minutes. 30 µL of sample was loaded in triplicates onto wells of a 12.5% polyacrylamide gels and run in a PROTEAN electrophoresis tank (Bio-Rad) at 100V for 15 minutes followed by 130V for 1 hour. Gels, filter paper and nitrocellulose membranes (Bio-Rad) were soaked in pre-chilled Towbin transfer buffer (25mM Tris, 192 mM Glycine, 20% methanol, pH 8.3) for 15 minutes. Transfer was performed using a Bio-Rad Trans-Blot semi-dry electrophoretic transfer cell (Catalog # 170-3940) by layering filter paper, nitrocellulose, gel and filter paper in a sandwich and running for 30

minutes at 15 V. Nitrocellulose membranes were washed in 1X TBST (137 mM NaCl, 20 mM Tris, 0.1% Tween-20) for 5 minutes and cut into strips at 50 kDa for β-Tubulin and 15 kDa for Galectin-1. The membrane strips were blocked in 1 x TBST + 5% skim milk for 30 minutes. The membranes were washed in 1 x TBST for 5 minutes and transferred into primary antibody for β-tubulin antibody rabbit IgG (sc-9104, Santa Cruz) at a ratio of 1:10000 and galectin-1 antibody (C-8) mouse IgG (Santa Cruz sc-166618) at a ratio of 1:500 diluted in 1 x TBST. Membranes were incubated in primary antibody on ice overnight. Membranes were then washed in 1X TBST for a total of 15 minutes on a shaker, changing fresh TBST every 5 minutes. The membranes were then transferred into secondary antibody and incubated for 3 hours on ice, anti-mouse conjugated to horse radish peroxidase (170-6516 Bio-Rad) for galectin-1 at a dilution of 1:10000 and anti-rabbit conjugated to horse radish peroxidase (170-6515 Bio-Rad) for β-tubulin at a dilution of 1:10000. The membranes were then washed again for 15 minutes in 1 X TBST changing to fresh TBST every 5 minutes. 200 µL of ECL (1705062 Bio-Rad) was added to each of the membranes just before imaging on a Chemidoc. Imaging was performed on a Chemidoc imager (BioRad) until the bands just reached saturation (1 second for β-tubulin and 30 seconds for Galectin-1). A white light exposure was also performed on each strip before both images were merged in order to match the ladder to the correlating bands.

3.3.4 Flow Cytometry

Flow cytometry analysis was performed on CRISPR gene edited HeLa cells as well as control HeLa cells as a secondary validation method. Cells were grown to 80% confluency in T-25 flasks and viability was checked using the trypan blue staining method mentioned above. Media was removed from the T-25 flasks and the cells were washed in 2 mL of 1 x phosphate buffered saline. 1 mL of Trypsin-EDTA (0.25%,

0.02%) was added and the cells were placed in a 35°C incubator for 5 minutes. Trypsinised cells were placed in a 15 mL tube and spun down in a centrifuge at 400 g for 4 minutes. Cells were gently vortexed while 2 mL of 70% ethanol was added dropwise. Cells were stored at 4°C for 24 hours to allow for fixation. Cells were then vortexed and spun down at 400 g for 4 minutes again and 70% ethanol supernatant was discarded. Cells were resuspended in 2 mL of 1 x PBS prior to flow cytometry analysis. Flow cytometry was performed on a BD FACSCanto II flow cytometer analyzing 10,000 events, using the following parameters: Forward side scatter (FSC) voltage: 240 V, Side Scatter (SSC) voltage: 300, B_530/30: 570 V, V_450/50: 475 V, V_510/50: 525 V. Data was collected and analysed using flowing software (Flowing software, 2013).

Chapter 4: Results

4.1 SWATH-MS host parasite interaction study

SWATH-MS analysis of host mammalian cervical epithelial (HeLa) cell samples consisting of 3 control T-25 flasks and 3 T-25 treatment flasks resulted in 2651 proteins quantified across all 6 samples. As shown below in Figure 6, of all the proteins quantified, a total of 22 proteins were identified as being differentially expressed in the treated group compared to the control. Proteins were considered as differentially expressed as either significantly upregulated or downregulated with a fold change of ± 1.5 (p value <0.05). Table 1 and Table 2 show a list of the 22 proteins that were significantly upregulated and downregulated, 8 proteins were identified to be upregulated while 14 were identified to be downregulated. Figure 7 shows a heat map highlighting that there was a lot of variation of up and down regulated proteins between each sample when clustered by euclidean distance.

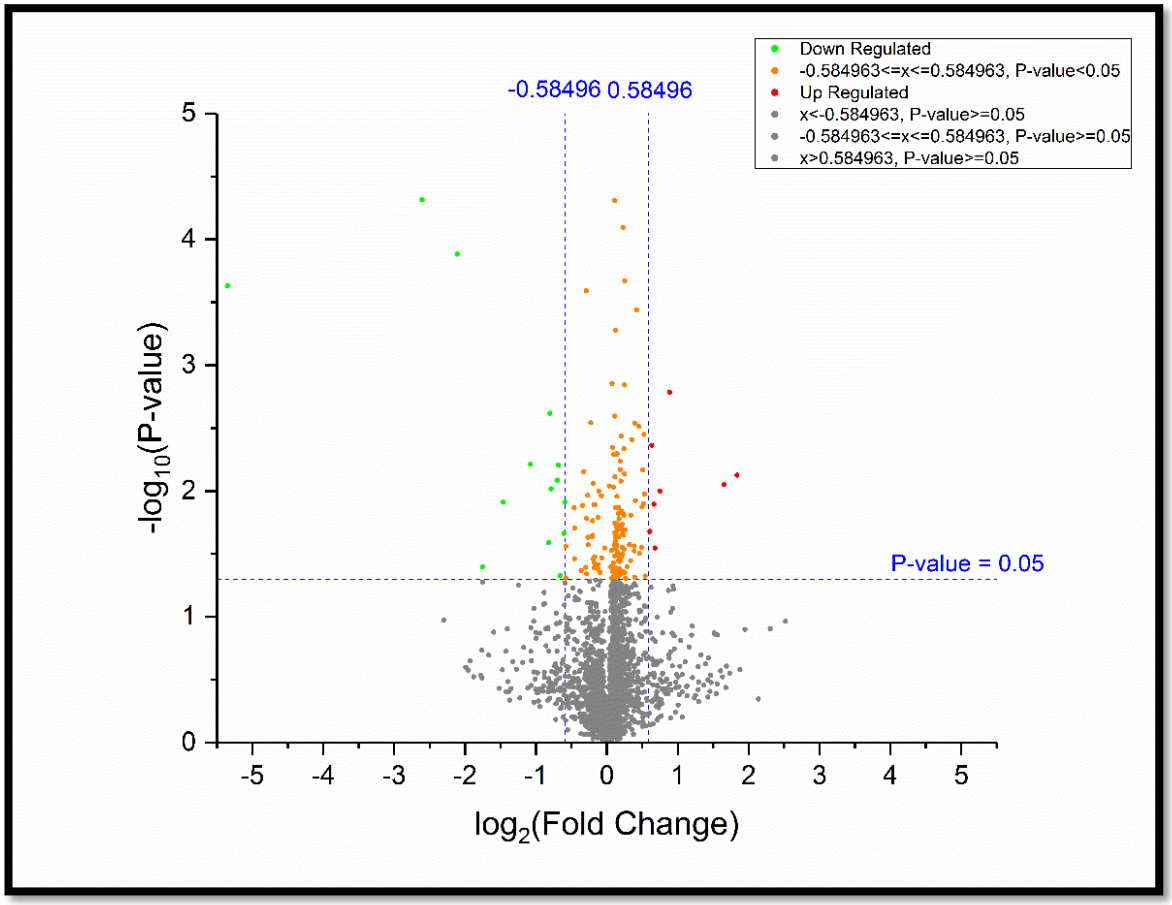

Figure 6. Volcano plot of all 2651 proteins identified in swath analysis, significant proteins with a –log10 P-value of <0.05 and a log2 fold change of > - 0.584963 were identified as being upregulated while a log2 fold change of > 0.584963 were identified as upregulated.

Table 1. List of all upregulated proteins identified in HeLa cells following interaction with *Trichomonas vaginalis*

Protein Name	UniProt Code	Protein Function	Fold Change	Up/Down Regulated
Periodic tryptophan protein 1 homolog	Q13610	Cell Growth, differentiation	3.569115	UP
Quinone oxidoreductase PIG3	Q53FA7	Oxidoreductase	3.147761	UP
von Willebrand factor A domain-containing protein 8	A3KMH1	ATPase activity	1.847819	UP
Serine protease HTRA2	O43464	Serine Protease, released during apoptosis	1.680249	UP
N-alpha-acetyltransferase 10	P41227	Acetylates proteins	1.605311	UP
AP-3 complex subunit beta-1	O00203	Protein sorting of transmembrane proteins targeted to lysosomes	1.584637	UP
Complement component 1 Q subcomponent-binding protein	Q07021	Involved in inflammation and mediates apoptosis	1.552889	UP
YLP motif-containing protein 1	P49750-4	Involved in the reduction of telomerase activity	1.524129	UP

Table 2. List of all downregulated proteins identified in HeLa cells following interaction with *Trichomonas vaginalis*

Protein Name	UniProt Code	Protein Function	Fold Change	Up/Down Regulated
Histone H2B type 2-E	Q16778	Component of the nucleosome, involved in chromosomal stability, DNA repair and replication	0.66425	DOWN
Putative RNA-binding protein 15	Q96T37	mRNA export factor	0.659121	DOWN
Ribosomal RNA processing protein 1 homolog B	Q14684	RNA processing	0.633589	DOWN
Lipase maturation factor 2	Q9BU23	Transmembrane protein involved in lipid metabolism	0.624055	DOWN
Alkyldihydroxyacetonephosphate synthase	O00116	Lipid biosynthesis	0.616785	DOWN
40S ribosomal protein S25	P62851	Component of 40S subunit of ribosomes	0.580483	DOWN
4-hydroxyphenylpyruvate dioxygenase-like protein	Q96IR7	Enzyme involved in tyrosine degradation	0.573365	DOWN
Exocyst complex component 4	Q96A65	Component of exocyst complex that targets vesicles for docking onto the plasma membrane	0.564916	DOWN
Tyrosyl-DNA phosphodiesterase 2	O95551	DNA repair enzyme	0.474183	DOWN

Solute carrier family 12 member 9	Q9BXP2	Subunit of a solute carrier family	0.362638	DOWN
Uncharacterized protein C11orf98	E9PRG8	Uncharacterised	0.29634	DOWN
Mitochondrial enolase superfamily member 1	Q7L5Y1	Catabolises L-fucose which is a carbohydrate attached to glycoproteins	0.232111	DOWN
Peptide deformylase	Q9HBH1	Deformylates nascent peptides regulating ribosomal regulation.	0.164544	DOWN
Ubiquitin carboxyl-terminal hydrolase 36	Q9P275	Transcriptional repressor by deubiquiting histones at the promoters of genes	0.024503	DOWN

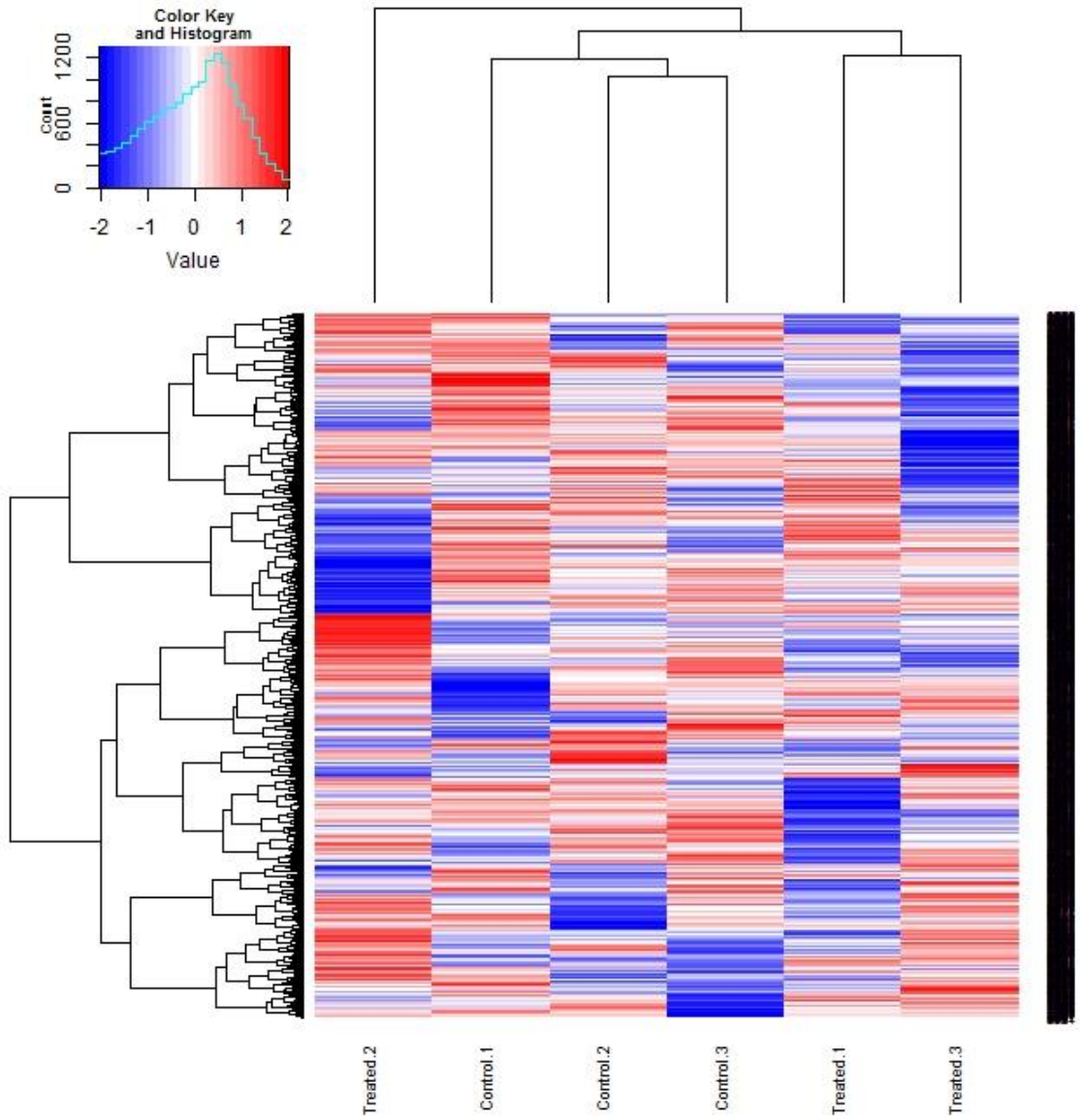

Figure 7. Heatmap of all quantified proteins clustered by Euclidean distance, Red = upregulated, blue = downregulated, white = no change. There is significant variation between the up and down regulated proteins in each sample, because of this variation the experiment should be repeated in an attempt to receive a more uniform trend in the results.

4.2 Puromycin Optimization

As shown in Figure 8, 2.5 µg of puromycin caused cell death to HeLa cells over the course of 72 hours. Cells were observed as rounded after selection begun, before the majority of cells detached at the 48-hour mark and leading to 100% cell death at the 72-hour mark. Figure 9 shows control wells over the same time period displaying increased growth, no cell death.

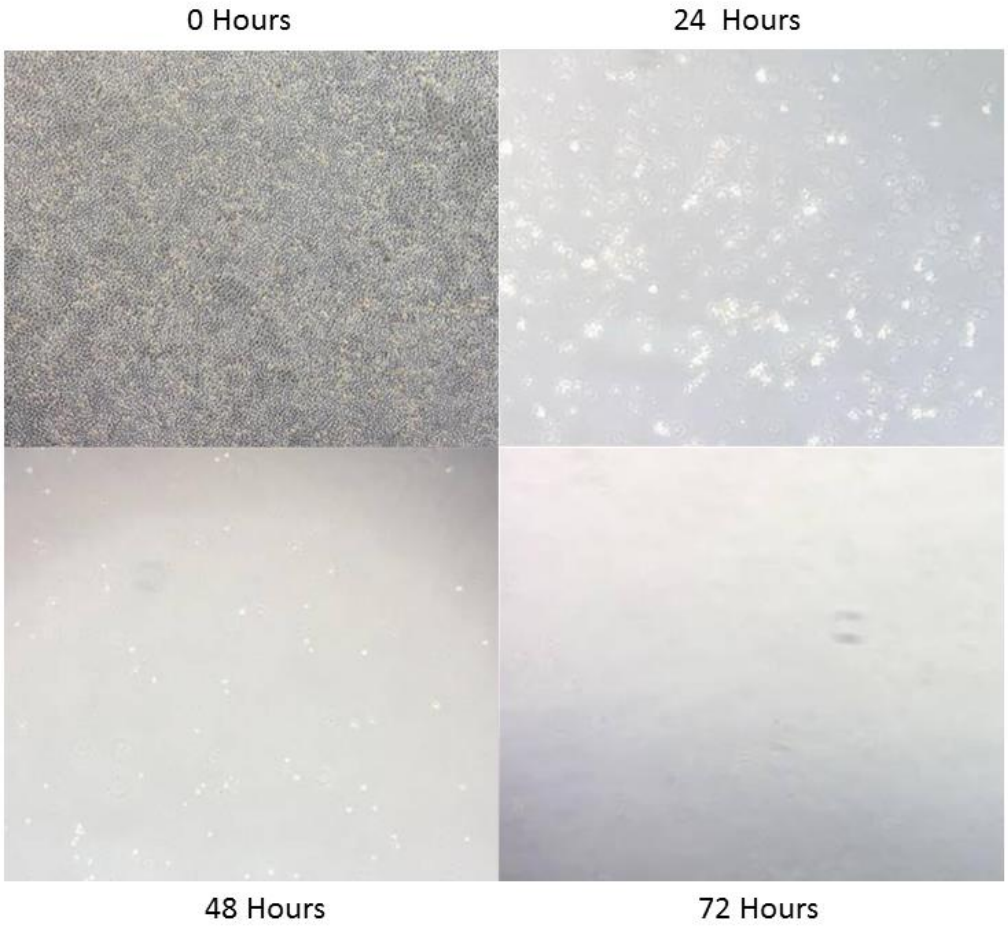

Figure 8. 2.5 µg of puromycin selecting HeLa cells over 72 hours in a 6 well plate. Image are of the 10x objective, by the 72 hour mark all cells have died.

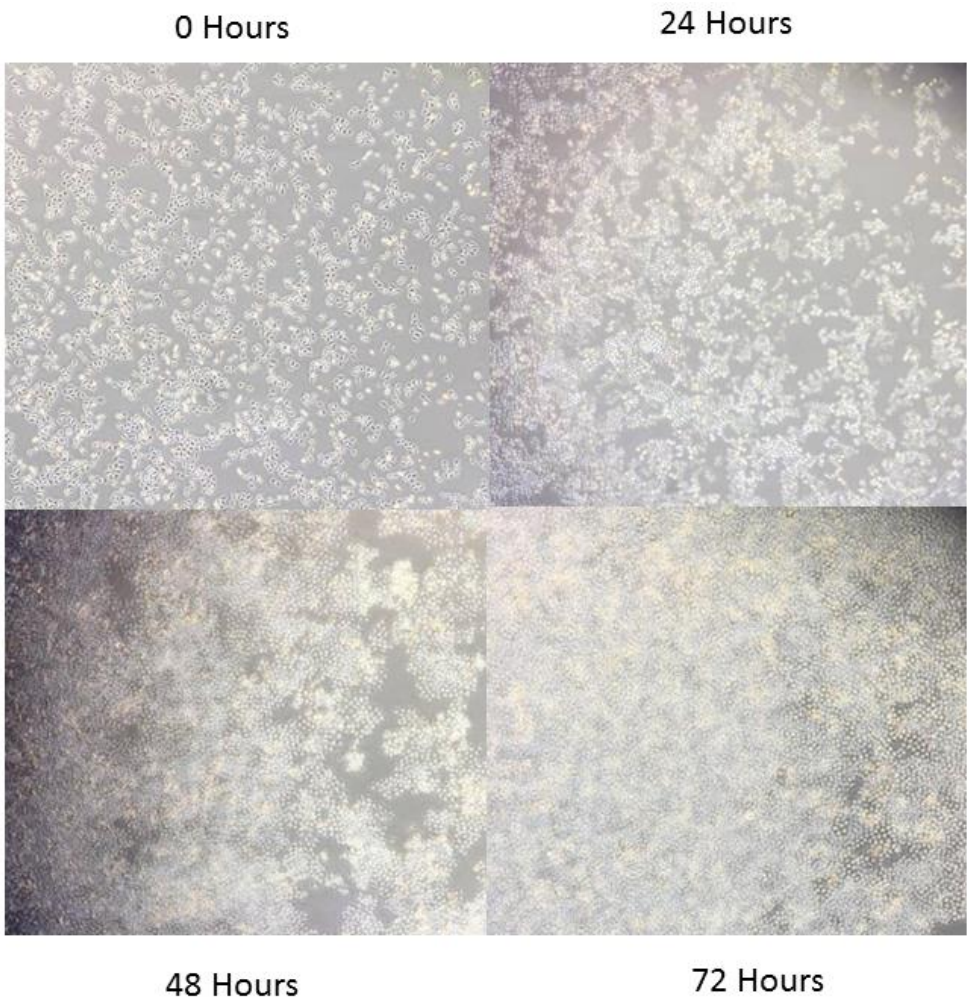

Figure 9. 0 µg of puromycin (Control) wells over 72 hours in a 6 well plate. Images are of the 10x objective, cells are seen to be growing and becoming overconfluent by 72 hours with no sign of cell death.

4.3 Galectin-1 Knockout

Western blot analysis was performed on HeLa cells that were treated in a 6 well plate with a dual nickase galectin-1 knockout plasmid for 6 hours, after selecting in 2.5 µg/mL of puromycin until all control cells were killed in control wells (72 hours). Figure 10 shows a reduction of galectin-1 expression in the treated wells when compared to the control wells. Densitometry analysis was performed on the average relative density of the western blots normalized to the loading control (β-tubulin).

The density of control blots produced a mean value of 90.97 while the mean treatment value was 51.35. A paired student t-test was performed finding a significant difference ($p < 0.05$) in the expression of galectin-1 between the control and treatment groups. Figure 11 shows there was a significant ($p < 0.05$) reduction of expression of galectin-1.

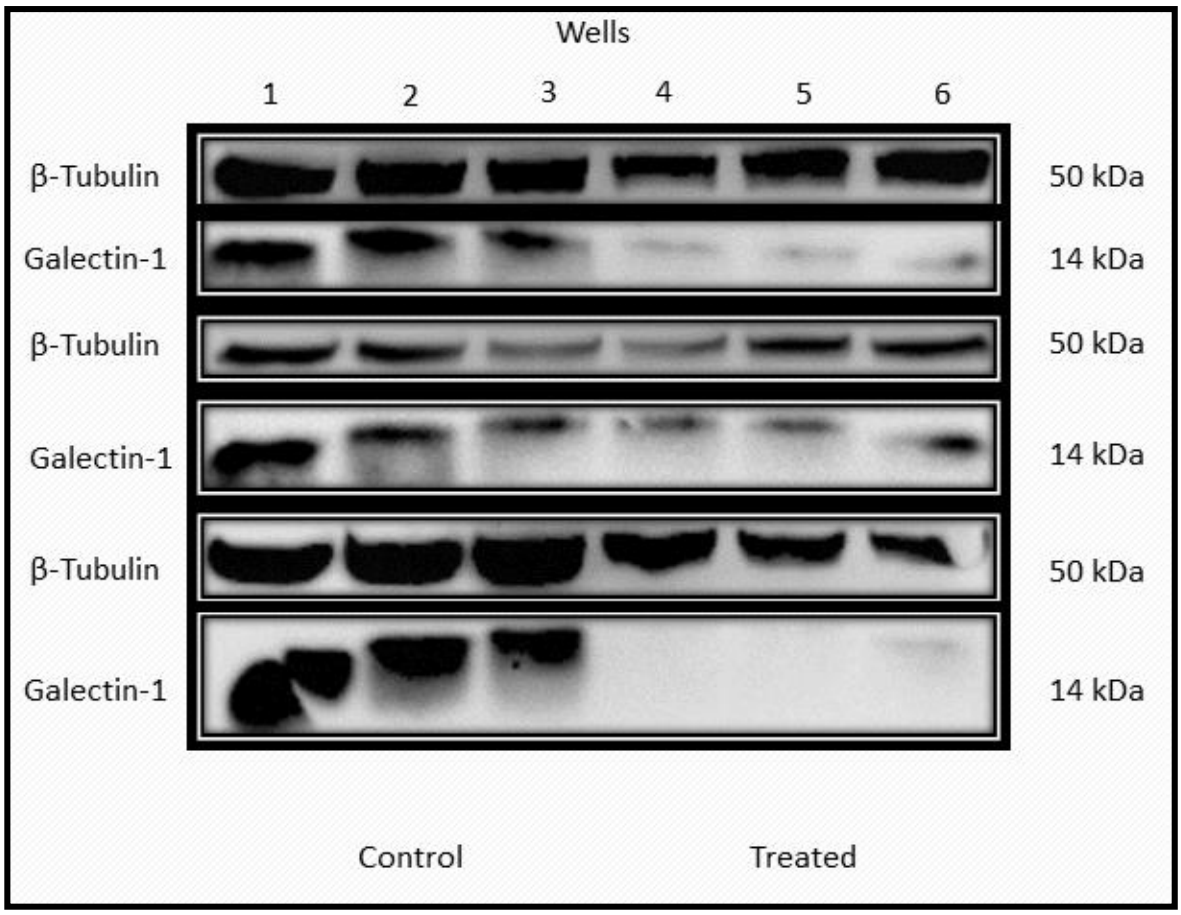

Figure 10. Western blots comparing control HeLa cells against HeLa cells that were treated with a Galectin-1 knockout. 30 µg of protein from control cells were loaded into wells 1-3 while 30 µg of protein from treated cells were loaded into wells 4-6. β-tubulin was used as a loading control for each western blot experiment. Experiment was performed in triplicates and images are of 3 independent replicates.

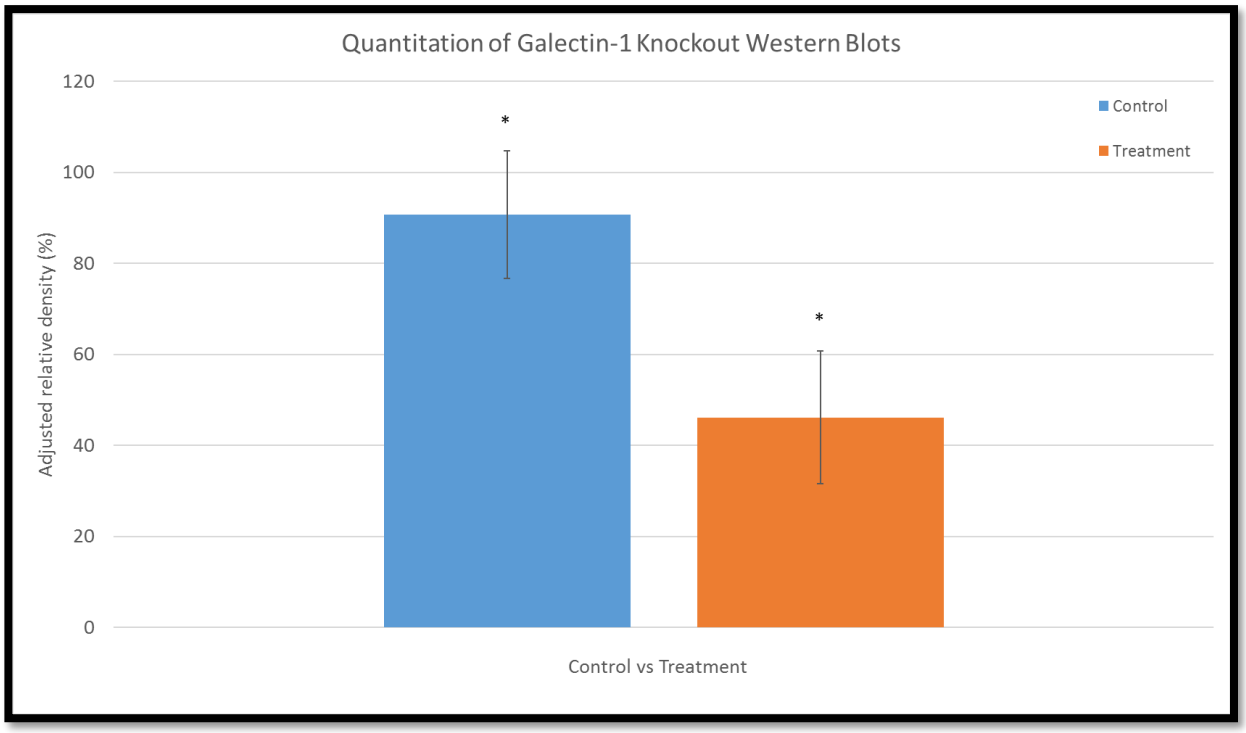

Figure 11. Densitometry analysis of the average of 3 independent replicate western blots comparing the relative density of each band normalized against β-tubulin (loading control). Average adjusted relative density of the control group was 90.7% while the average adjusted relative density of the treatment group was 46.1%. A paired students t-test was performed finding a significant difference (p <0.05) against the control vs treatment group.

4.4 Flow Cytometry

Figure 12 shows flow cytometry analysis of green fluorescent protein (GFP) expression in Control cells and knockout cell populations analyzing 10,000 events. Both control and knockout groups fluoresce when using a 530/30 nm emission filter. There was a slight difference in the mean fluorescence of both groups with a mean fluorescence average of 3490.47 in the control group and 4324.49 in the treatment group.

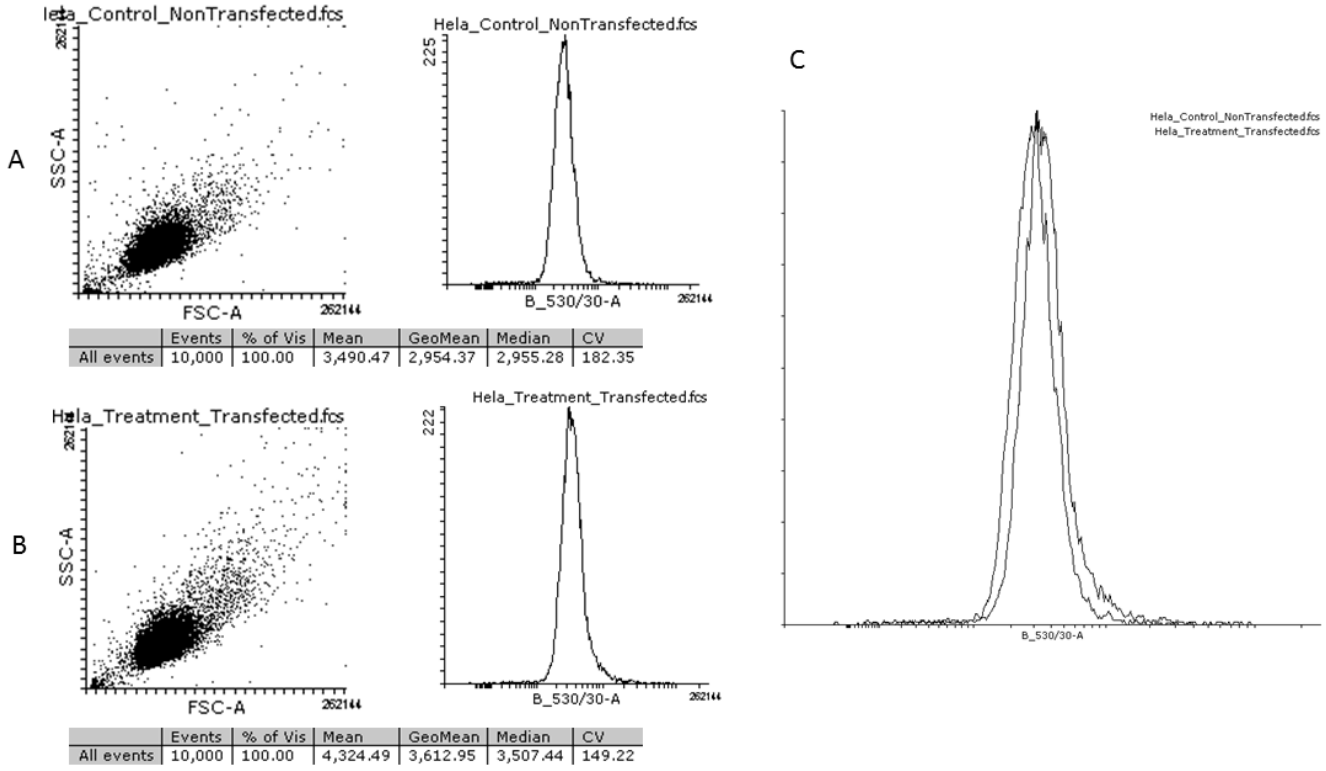

Figure 12. Flow cytometry analysis of GFP fluorescence at 530 nm, of control HeLa cells (A) vs transfected HeLa cells (B). Histograms of both groups were superimposed to compare the differences between each group (C). Images show fluorescence in both the control (HeLa non transfected) and treated groups at 530 nm with a slight shift in the mean from 3,490 RFU in the control to 4,324 RFU in the treated group.

Chapter 5: Discussion

5.1 Host-Parasite interaction study using SWATH-MS

The aim of the host parasite interaction study was to use SWATH-MS to identify whether the presence of B7RC2 strain *T. vaginalis* could influence protein expression within host cells which may play a key role in the infection process. As shown in Figure 6, there was a total of 2651 proteins identified in the SWATH-MS analysis. Of the 2651 proteins, a mere 22 were identified as being differentially expressed i.e. significantly upregulated and downregulated in the treatment group (interacted) compared to the control. Proteins were considered to be differentially expressed, either upregulated or downregulated, with a fold change of ± 1.5 and a students t-test *p* value <0.05. Table 1 shows a total of 8 upregulated proteins with fold changes ranging from 3.57 to 1.52 at the protein level. Table 2 shows a total of 14 downregulated proteins with fold changes ranging from 0.66425 to 0.024503.

Periodic tryptophan protein 1 homolog (Pwp1) was the most upregulated in the interaction study. Found in the nucleus, Pwp1 is encoded by the *PWP1* gene and consists of several WD40 repeat proteins. WD40 proteins are abundant throughout the human proteome and have been implicated in many biological functions from regulation of transcription, apoptosis and signal transduction (Xu & Min 2011). Pwp1 has been found to play a role in growth and differentiation. A study by Shen et al (2015) found that Pwp1 expression is needed to differentiate mouse embryonic stem cells (mESC) from pluripotent state to all other cell lineages. Shen et al (2015) found that knocking down *PWP1* affects differentiation but not proliferation or apoptosis (Shen et al. 2015). The *PWP1* gene is also associated with regulating nutrient dependent growth through ribosomal gene expression by acting in tandem with the mTOR signaling pathway to regulate transcription factor 2 (Liu et al. 2017). Liu et al. (2017) found high levels of Pwp1 was associated with a poor

prognosis in head and neck squamous cell carcinoma due to the higher rate of ribosome biogenesis leading to more aggressive tumors (Liu et al. 2017). The high upregulation of Pwp1 in HeLa cells after interaction with *T. vaginalis* poses an interesting question; is *T. vaginalis* sabotaging its host by signaling as a potential nutrient in order to stimulate cellular proliferation? Studies have shown that *T. vaginalis* secretes molecules such as *T. vaginalis* macrophage migration inhibitory factor (TvMIF), a pro inflammatory cytokine, that promotes inflammation and cellular proliferation which may be implicated in an increase in prostate cancer risk (Mitteregger et al. 2012; Twu et al. 2014). According to Twu et al. (2014) TvMIF mimics human macrophage migration inhibitory factor (HuMIF) and stimulates CD74 binding leading to the activation of the Akt/BAD pathway. Downstream of the Akt pathway is the mTOR signalling pathway as shown in Figure 13 that Pwp1 is a member of, although TvMIF and CD74 are not directly implicated in *PWP1* upregulation it may potentially be affected downstream (Liu et al. 2017; Twu et al. 2014). As HeLa cells are a cancerous cell line it may potentially be affecting the upregulation of Pwp1, Future *T. vaginalis* interaction studies could look more at the role of host Pwp1 and why it may be upregulated, using a primary cell line to distinguish whether the upregulation was due to *T. vaginalis* of it was due to the cancerous cell line.

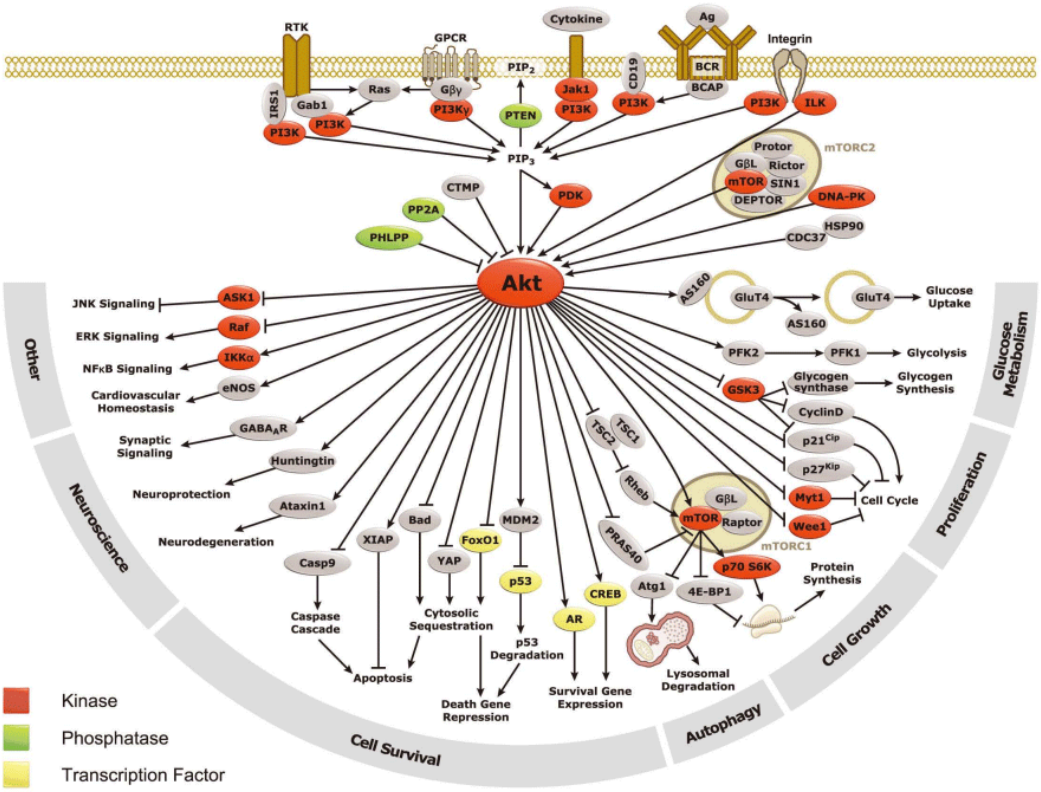

Figure 13. AKT cell signalling pathway and associated downstream pathways including the mTOR pathway that Pwp1 is a part of, affecting cellular proliferation as well as inflammation. (Bio-Connect 2008).

Interestingly the 40S ribosomal protein S25 which is a protein that makes up a component of the 40S ribosome subunit was found to be downregulated yet it was the only protein component of the ribosome to be differentially expressed. 40S ribosomal protein S25 is one of 80 distinct proteins that make up the two ribosomal subunits which have function as not only ribosome assembly and translation but also have independent functions (Zhou et al. 2015). Ribosomal protein S25 is encoded by the gene *RPS25* and has been implicated in regulating cell signaling pathway MDM2-p53 by suppressing cell proliferation. *RPS25* regulates the ubiquitination of p53 a tumor suppressor by binding to MDM2 preventing p53 from being degraded. When cells are in ribosomal stress, which may occur due to the cytotoxic effects of *T. vaginalis*, *RPS25* is downregulated in the cytosol leading to the

degradation of p53 and thus increased cell proliferation (Zhang et al. 2013). It is not clear whether the interaction of *T. vaginalis* with HeLa cells would have caused this downregulation in order to increase the proliferation of cells. It is more likely that this is due to HeLa cells being a cancer cell line, and the increased stress caused by the parasites lead to this downregulation naturally due to the cancerous nature of the cell line and the affected protein pathways (Leroy et al. 2014). Further studies using non-cancerous cell lines could be performed to look further into whether the downregulation of Ribosomal protein S25 is biologically relevant (Leroy et al. 2014).

The second most upregulated protein compared to control cells was Quinone oxidoreductase PIG3 encoded by the gene *TP53I3.* Quinone oxidoreductase PIG3 is an enzyme with oxidoreductase activity that is mediated by the tumor suppressor p53, responding to cellular stresses such as oxidative stress (Pospisilova et al. 2004). When *T. vaginalis* interacts with host cells, it is able to stimulate the production of proinflammatory host cytokines such as IL-1, IL-6, and TNF-α as well as nitric oxide (Han et al. 2009). The increase of nitric oxide and cytokines causes stress on the mammalian cells which can activate apoptotic pathways such as the expression of Quinone oxidoreductase PIG3 mediated by p53 which leads to cell apoptosis (Pietsch et al. 2008). The high upregulation of Quinone oxidoreductase PIG3 may be due to the response to damage by *T. vaginalis* and the cellular environment activates pathways to kill the respective cells that may be beyond repair. Of the proteins identified in this host-parasite interaction study, many were related to damage and repair of the mammalian epithelial cells such as the upregulated serine protease HTRA2, complement component 1 Q subcomponent binding protein (gC1qR) and the downregulated histone H2B type 2-E.

Serine protease HTRA2 is an enzyme encoded by the *HTRA2* gene and is involved in apoptosis. This protease is released in the cytosol where it then contributes to cell death via apoptosis (Vande Walle, Lamkanfi & Vandenabeele 2008). HTRA2 can induce caspase activity by removing the inhibitors of caspases 3, 7 and 9 through its own proteolytic activity. By removing the caspase inhibitors such as XIAP which is bound to caspases 3,7 and 9 inhibiting their activity, these caspases become enzymatically active leading to apoptosis and cell death (Yang et al. 2003). The upregulation of HTRA2 may be due to *T. vaginalis* cytotoxic effects. A study by Gilbert et al. (2000) suggest that *T. vaginalis* produces contact depended cytotoxic effects including the induction of apoptosis. Further studies by Lustig et al. (2013) have shown that cytolysis of both prostate and vaginal epithelial cell lines seem to need *T. vaginalis* to be attached in order to produce cytolysis of the cells (i.e. contact dependent). Lustig et al. (2013) blocked the attachment of *T. vaginalis* to epithelial cells by the addition of a 0.4 µm porous membrane which allowed any secreted proteins to pass through and found that this blocked the cytolysis of the epithelial cells (Lustig et al. 2017). Studies have also shown protease activity is able to initiate apoptosis in host cells. The addition of E-64, a cysteine protease inhibitor, was able to inhibit apoptosis of vaginal epithelial cells. This is significant because it is well established that *T. vaginalis* secretes several proteases including cysteine proteases including CP30 fraction which consists of CP2, CP3, CP4 and CPT CP2, CP12, CP39, and rhomboid proteases (Figueroa-Angulo et al. 2012; Kummer et al. 2008; Riestra et al. 2015; Sommer et al. 2005). It is likely that the release of these cysteine proteases was initiating apoptosis, since the study was performed on HeLa cells in a medium of 1:1 DMEM: DMM, there iron would have been available in the media. One study by Sommet et al (2005) proved that the release of some cysteine proteases from *T. vaginalis* is

modulated by iron availability, reducing the amount of iron increases the release of CP30 in order to induce apoptosis and inflammation which allows for the uptake of nutrients from the host cells. Future studies could look at reducing or increasing iron availability while the interaction is performed to see if that has an effect on what proteins are upregulated or downregulated in those different environments (Sommet et al. 2005).

Complement component 1 Q subcomponent binding protein (gC1qR) was upregulated and is a protein that is involved in numerous biological functions ranging from ribosome biogenesis, apoptosis regulation, and infection/inflammation processes. gC1qR is a binding protein involved in the complement system which can trigger chemotaxis and phagocytosis. gC1qR can bind to the first component of the complement system C1q to stimulate the movement/infiltration of neutrophils (Leigh et al. 1998). GC1qR is expressed both in on the surface of the cell as well as intracellularly and can induce the production of pro-inflammatory factors such as the activation of the complement system as well as generating kinins such as bradykinin to dilate blood vessels and promote coagulation (Ghebrehiwet et al. 2006). Interestingly this protein has been implicated to be exploited by many pathogens including viruses, bacteria and parasites such as hepatitis C, HIV, *Staphylococcus aureus, Listeria monocytogenes* and *Plasmodium falciparum* (Braun, Ghebrehiwet & Cossart 2000; Fausther-Bovendo et al. 2010; Nguyen, Ghebrehiwet & Peerschke 2000; Pednekar et al. 2016). Pathogens exploit gC1qR to either suppress the immune response which increases the likelihood of survival of pathogens or they utilize it to crosstalk with the host cells. For example, hepatitis C virus uses a core protein HCV to bind to host GC1qR. This suppresses the ability for T cells to proliferate thus reducing the impact of the natural immune response against this virus (Pednekar et

al. 2016). *L. monocytogenes* uses host GC1qR as a receptor for InlB, a surface protein, to induce signals and allow cross talk and initiate internalization (Cossart, Pizarro-Cerda & Lecuit 2003). *P. falciparum* adheres to cell surface GC1qR via a domain on the protein called *P. falciparum* erythrocyte membrane protein 1 (Magallón-Tejada et al. 2016). Currently there are no studies exploring the interaction of *T. vaginalis* and GC1qR yet based on interactions with other parasites and pathogens, there could be a potential for *T. vaginalis* to be utilizing cell surface GC1qR to adhere or to promote inflammation. Future experiments could look at GC1qR upregulation to verify whether it is consistently upregulated and perform a knockout using CRISPR/cas9 and see if it effects *T. vaginalis* attachment to host cells.

Another protein that was downregulated and related to damage and repair of the host cells was Histone H2B type 2-E. Histone type 2-E is a protein that is involved in the maintenance of chromosomes by compacting chromatin as well as the repair of DNA (Chen-Yi et al. 2011). Histone type 2-E has been proposed to have anti-microbial/fungal effects on bacteria and fungi as well as against the parasite *Leishmania* through modulation of the immune response, acting as an antimicrobial peptide (Kawasaki & Iwamuro 2008; Wang et al. 2011). As histone type 2-E was found to be downregulated in the host cells there is a potential interaction where *T. vaginalis* is suppressing histone type 2- E in order to aid its survival by suppressing the innate immune response. Further studies into how histones affect and interact with *T. vaginalis* could be performed in the future.

The third most upregulated protein was von Willebrand factor A domain- containing protein 8 (VWA8). Unfortunately, there is not much known about this protein. A study by Luo et al (2017) attempted to characterize this protein in mouse liver cells and found that

it has ATPase activity and functions as enzymes that import metabolites in cellular metabolism as well as exporting any toxins out of the cell (Luo et al. 2017). As there is little research available on the function of VWA8 it is hard to discern its function in relation to the hosts interaction with *T. vaginalis*. It may be that it is being upregulated to export toxins out of the cell created from *T. vaginalis* release of proteases, such as cysteine proteases, metalloproteases and rhomboid proteases which function to lyse the host cells (Cardenas-Guerra et al. 2013; Quintas-Granados et al. 2013; Riestra et al. 2015). Further studies will need to be performed to understand the full function of VWA8 and whether it is consistently upregulated in response to *T. vaginalis.*

N-alpha-acetyltransferase 10 (Naa10) was upregulated which is a protein that forms part of a complex called NatA acetyltransferase. NatA acetyltransferase is an enzyme that adds acetyl groups to proteins formed by ribosomes. Around 80% of proteins found in humans are acetylated at the N-terminus (front end) of the protein while 40% are specifically acetylated by the Naa10 subunit (Dörfel & Lyon 2015; Foyn et al. 2013). The Naa10 subunit has many functions including regulating DNA damage repair responses and apoptosis, inducing cellular death in response to damage to DNA (Yi et al. 2011; Yi et al. 2007). As Naa10 was upregulated in response to *T. vaginalis*, and given that a large number of proteins are acetylated, Naa10 is most likely upregulated in order to process other proteins that have become or are becoming expressed due to the interaction. Naa10 may also be upregulated in response to damage by the parasite as part of the process of apoptosis.

AP-3 complex subunit beta-1 (AP3B1) was upregulated which is a subunit of a protein involved in protein sorting, especially proteins in organelles and lysosomes

(Dell'Angelica, Ooi & Bonifacino 1997). AP3B1 interacts with clathrin which forms coated vesicles (Drake, Zhu & Kornfeld 2000).Vesicles play many roles including nutrient transfer, cell signaling, and the mediation of the immune response. AP3B1 is a component of the AP3 complex that is required for the trafficking of Toll like receptors such as TLR2, TLR4 and TLR9 as well as the activation of interleukin 6, a pro inflammatory cytokine, as part of the innate immune response (Petnicki-Ocwieja et al. 2015). The upregulation of AP3B1 is most likely due to the innate immune response to *T. vaginalis* in an attempt to destroy the parasite. IL-6 has been found to be upregulated by *T. vaginalis* to produce an inflammatory response, as well as other pro inflammatory mediators such as IL-8, IgA, IgG and Th1 (Han et al. 2009; Fichorova 2009). *T. vaginalis* selectively upregulates these mediators potentially to exploit and evade the immune system by creating a chaotic microenvironment within the host cells. Further studies could be performed to see the exact relationship between AP3 and its compartments and *T. vaginalis* to see whether the upregulation of AP3 does in fact aid *T. vaginalis* or whether it is just the natural response of the host bodies towards the parasite. Studies could include generating a knockout of AP3 and interacting the knockout with *T. vaginalis* parasites and studying the impact on the immune response.

Many of the downregulated proteins seem to be affecting the cell's ability to mature proteins including Putative RNA-binding protein 15, Ribsomal RNA processing protein 1 homolog B, Lipase maturation factor 2, alkyldihydroxyacetonephosphate synthase, Tyrosyl-DNA phosphodiesterase 2, solute carrier family 12 member 9, peptide deformylase and ubiquitin carboxyl- terminal hydrolase 36. These proteins are normally involved in protein synthesis, lipid metabolism and biosynthesis, RNA synthesis and the

exportation of mRNA. The downregulation of these proteins is most likely due to stress responses on the cells as well as the initiation of apoptosis. Studies have shown that stress to cells causes the cells to inhibit protein synthesis either to conserve energy and limit the production of proteins to the most vital in order to survive the adverse environmental conditions or due to the activation of apoptosis as the cells need to redirect energy to the activation of caspases leading to cell death. As shown in Figure 14, there is a balance between cell responses to damage, balancing survival and apoptosis through signaling cascades leading to survival or cell death (Elmore 2007; Nowsheen & Yang 2012; Peng et al. 2007).

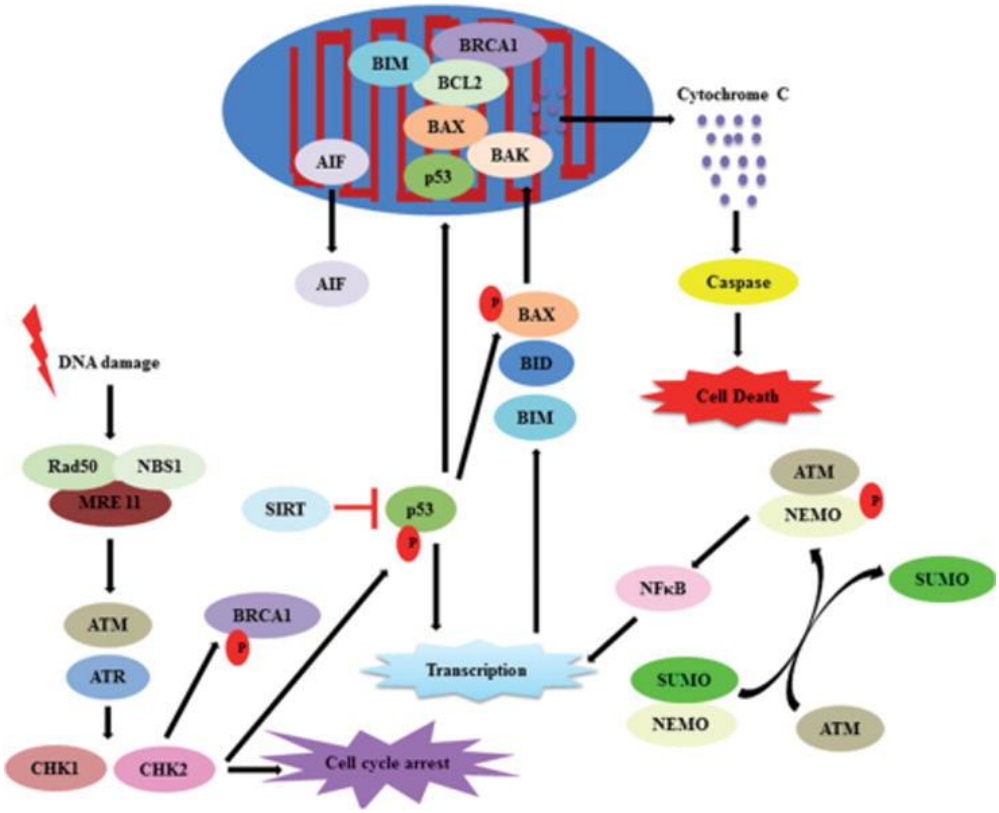

Figure 14. Signaling cascades leading to cell death, cell cycle arrest or transcription of survival genes. Host cells balance cell cycle arrest, transcription and cell death based on the signaling environment, when caspases are released due to these signals it leads to cell death (Nowsheen & Yang 2012).

In summary, many of the proteins that were differentially expressed in this host-parasite interaction study relate to apoptosis and the induction of immune responses (Serine protease HTRA2, Pwp1, Quinoneoxidoreductase PIG3, Naa10, AP3B1 and Histone H2B type 2-E) while a small group of proteins seemed to effect proliferation and potential attachment of the parasite (gC1qR, Pwp1). The remainder appeared to impact the maturation of proteins in response to cellular stress from the parasite. The results have generated further questions that could be used in future studies. As the interaction was performed for 30 minutes, it could be extended further to see if the protein profile changes even more following the establishment of infection. Further studies could also be performed on Pwp1 to see if *T. vaginalis* does try to initiate proliferation in the mammalian cells. Further studies on the protein gC1qR could also be performed to see if *T. vaginalis* uses this protein to attach similar to other parasites mentioned above.

As Figure 7 shows, there was a lot of variation between the samples, in terms of upregulated and downregulated proteins. This effects the end result as there seems to be many proteins being differentially expressed in all the samples but due to the large variation they are not statistically significant, with only 22 proteins that were consistently differentially expressed between the control and treatment groups. This experiment should be repeated again with 3 independent replicates in order to gain more accurate results.

Interestingly galectin-1 was identified in the SWATH interaction study but was not identified as being differentially expressed (Data in appendix) this could be due to a few reasons, galectin-1 is found to be abundant throughout the cell, on the cell surface, in the cytoplasm and within the nucleus so *T. vaginalis* may not necessarily need to upregulate galectin-1 to use it as an attachment point onto host cells (Camby et al. 2006) The

interaction time may also have been too short to potentially see changes in galectin-1 expression across the cell and increasing the amount of time *T. vaginalis* is allowed to interact with the cells may start to show changes in galectin-1 expression.

5.2 SWATH-MS interaction study methodology

The Swath interaction study was performed for 30 minutes and 5 x 10^4 parasites/ ml of parasites were chosen as a baseline with the view to increase the time as well as concentration of parasites in further SWATH experiments. 5 x 10^4 parasites/ ml (Ratio of 1:5 parasites/ HeLa cells) was chosen as a baseline as many previous works have used ratios ranging from low (1:5) all the way to high (30:1) (Dias-Lopes et al. 2017; Kim et al. 2017; Salvador-Membreve, Jacinto & Rivera 2014; Vilela & Benchimol 2011). Due to the variation of attachment ratios and times, the interaction time and the number of parasites was chosen based on previous literature. A study by Kucknoor, Mundodi & Aldarete (2005) studying the differential expression of genes in vaginal epithelial cells after interacting *T. vaginalis* concluded after optimizing an adherence assay, that a 30-minute interaction time was the most ideal. Kucknoor, Mundodi & Aldarete (2005) found that *T. vaginalis* would adhere to the cell monolayer within 15 minutes, however, at 45 minutes disruption of the monolayer and significant cytotoxicity would occur. Many other studies vary in their interaction times ranging from 30 minutes to days. Due to this fact, 30 minutes was chosen with the view to increase the interaction time in future experiments (Dias-Lopes et al. 2017; Kim et al. 2017; Salvador-Membreve, Jacinto & Rivera 2014; Vilela & Benchimol 2011). A low parasite to cell ratio was chosen with the view to decrease the cytotoxic effects of the parasites on the cells, for example using a high

number would increase the number of proteases released causing damage to the cells at a faster rate and impacting what proteins would be seen as upregulated or downregulated.

A study by Lustig et al (2013) that looked at contact dependent cytolysis of epithelial cells and interacted 23 different strains of *T. vaginalis* found that there was a significant amount of variation of attachment and cytolysis between the strains. Lustig et al (2013) also found that there was a significant correlation between lowly adherent *T. vaginalis* strains and higher cytotoxity but higher adherent strains did not see this same correlation. The parasite that was used in this experiment was a highly adherent strain (B7RC2) while a colleague used a lowly adherent strain (PRA98) in tandem however due to a budget constraint only 9 samples could be performed, 3 B7RC2, 3 PRA98 and 3 controls, based on the increased cytotoxity the lowly adherent strain would have on the cells the lowest time point was used. Interestingly many more proteins were found to be differentially expressed in the colleague's strain (Data shown in appendix). Using a 30-minute interaction time and an MOI of 1:5 parasites/ HeLa cells this experiment could be used as a baseline with a view of increasing time and number of parasites in further experiments which would give a greater view on what host proteins are differentially expressed from the initiation of attachment (30 minutes) to much longer times.

HeLa cells were chosen as they were the only available vaginal epithelial cells, VK2 E6E7 cells were initially going to be used but were unable to be revived and due to budget and time restrictions no more could be acquired. HeLa cells were then chosen as a backup as they were available and have been previously used in other *T. vaginalis* studies. HeLa cells have been used in many host parasite interaction studies involving *T. vaginalis* from studies performed in 1985 that looked at *T. vaginalis* attachment until now where more

recent studies looking at drug candidates that may be active against *T. vaginalis* (Alderete & Garza 1985; Mandalapu et al. 2016; Vieira et al. 2016). In future experiments, it would be ideal to use another cell line such as VK2/E6E7 as they are closer to the legitimate environment that *T. vaginalis* would live in as these cells are vaginal epithelial cells while HeLa are cervical epithelial cells (Fichorova et al. 2016). Interacting *T. vaginalis* with a prostate epithelial cell line would also be ideal due to the fact that *T. vaginalis* infects both male and females.

5.3 Galectin-1 Knockout

The aim of the galectin-1 knockout proof of principle study was to generate a complete knockout of galectin-1 to use in future experiments. As shown in the Figures 10 and 11, the complete knockout of galectin-1 was unsuccessful. As Figure 10 demonstrates, there was a reduction of galectin-1 expression following western blot analysis. Although there is a reduction, partial expression is still revealed of galectin-1 after performing the CRISPR knockout. Densitometry was performed on the western blots in order to quantify whether there was a significant reduction of galectin-1 when normalized to a loading control (β-tubulin). As Figure 10 shows, there was a significant reduction of the expression of galectin-1 with an adjusted relative density for the control group of 90.7% and 46.1% for the treatment group respectively. A paired t-test was performed on the average of the triplicate western blots which showed there was a significant difference between the two groups ($p < 0.05$). This shows there is a reduction of galectin-1 expression within the HeLa cell population but galectin-1 is still expressed either at a lower amount or in some cells in the population. There are multiple factors that may be at play as to why

a complete knockout of galectin-1 was unable to be produced. These may include the CRISPR plasmid used, technique, available equipment and wild type contamination. When attempting a complete knockout of a gene, the sequence of the CRISPR gRNA is an important factor in whether the product will be able to generate a complete knockout. The gRNA sequences determine where insertions or deletions will occur, directing Cas9 to the target and introducing a double strand break. The sequence must also be adjacent and upstream of a protospacer adjacent motif (PAM) (Cencic et al. 2014). For a complete knockout the sequences must delete part of an exon causing a frameshift mutation after non homologous end joining repair pathway, that will cause the protein to be misfolded and degraded or by destroying the exon/ intron junction so that the protein may not be expressed (Bauer, Canver & Orkin 2015). If the sequences completely splice out an exon but the open reading frame remains intact, isoforms of the protein may then be produced which may be detected by the galectin-1 antibody. The guide RNA sequences in the dual nickase CRISPR plasmid used in the knockout for LGALS1 (sc-400941-NIC Santa Cruz) were GGAGAGTGCCTTCGAGTGCG and AGGTTTGAGATTCAGGTTGC. When querying the sgRNA sequences using E-CRISP (Heigwer et al. 2014) to validate the sequences it was found that AGGTTTGAGATTCAGGTTGC targets the negative sense strand with a specificity score of 40, annotated score of 54.6 and an efficiency score of 33. The Doench et al (2014) score was 0.024 and Xu et al (2015) score was 0.0734229352. GGAGAGTGCCTTCGAGTGCG targets the positive sense strand with a specificity score of 40, annotated score of 52.8 and an efficacy score of 70.3. The Doench et al (2014) score was 0.56 and Xu et al (2015) score was 0.2551392616. According to the queried results, despite the low efficiency and specificity score, the sgRNA should be targeting all 4 exons leading to a complete knockout of galectin-1. The Doench scores gives a score based on

on-target effects to calculate how well an sgRNA will function at its specific site. The higher the Doench score and Xu score the higher the chance of the sgRNA targeting its specific site with higher efficiency and less off target effects. When screening LGALS1 for sgRNA sites using E-CRISP evaluation tool there were many sgRNA sequences that had higher Doench and Xu scores while also having higher specificity, annotated and efficacy scores. For example, the minus sense strand CACAAGCCATGATTGAGTCC specificity score of 40, annotated score of 81, efficacy score of 37 plus Doench score of 0.084 and a Xu score of 0.267. This would be a better sgRNA sequence to use in place of the lower scoring minus sense strand used in the pre made Santa Cruz product and designing and making our own plasmid may produce better results although it would require a longer process of design and validation of the sequences and plasmid (Heigwer, Kerr & Boutros 2014).

There are factors other than the product that also could be causing the incomplete knockout such as the techniques used. The knockout of galectin-1 on mammalian HeLa cells was performed in accordance to the protocol provided by Santa Cruz, first by optimizing puromycin amounts to obtain the lowest concentration of puromycin that kills 100% of non-transfected cells in 3-5 days. Figure 8 shows the use of 2.5 µg of puromycin which was able to kill 100% of the cells within 72 hours compared to a control plate of 0 µg shown in figure 9. Secondly transfection reagent amounts were optimized, when performing initial experiments, the cells would all die higher than 5 µl of transfection reagent. A similar problem occurred when using higher than 1 µg of plasmid DNA so that was chosen as a baseline. For the amount of time to transfect, it was found that following Santa Cruz' protocol to transfect for 24-72 hours would also kill all the cells so the time

was reduced until cells were seen to be alive (6 hours of transfection). The number of cells that did survive was minimal after transfection, with only about 10% of the initial cells surviving. Puromycin selection was then performed on these cells, in conjunction with control wells and puromycin selection was not stopped until 100% of the cells in the control wells were dead. The cells were left to grow to confluency which took a few weeks of slow growth before there was enough to expand to T-25 and T-75 flasks to perform western blot analysis. Flow cytometry was also utilized as the plasmid has both a green fluorescence protein (GFP) marker and puromycin marker. As shown in Figure 12, HeLa seems to auto fluoresce at the same wavelength of GFP used in the experiment (530 nm) There was a slight difference in the mean fluorescence of both groups with a mean fluorescence average of 3490.47 in the control group and 4324.49 in the treatment group meaning there was a slight increase in fluorescence caused by GFP between the control and treatment groups but we could not use this data to accurately confirm all cells in the population were transfected. Due to a lack of a cell sorter, sorting of GFP expressing cells could not be performed which would of greatly increased the chances of selecting only positively transfected cells (Bauer, Canver & Orkin 2015). Normally PCR would also be performed on the cells to confirm the knockout on a genomic scale and to positively select for 100% homozygous clones where all the alleles of the target gene have been edited, as there may be a heterozygous population of cells. Due to a restricted budget only western blotting was used but PCR should be used in conjunction in future knockout attempts. If the population are heterozygous there will still be an expression of galectin-1 which would be detected by western blotting. As Figure 15 shows, PCR or mismatch cleavage assays can be used to positively select for homozygous populations, PCR product should decrease if the cells are positively edited (Ran et al. 2013).

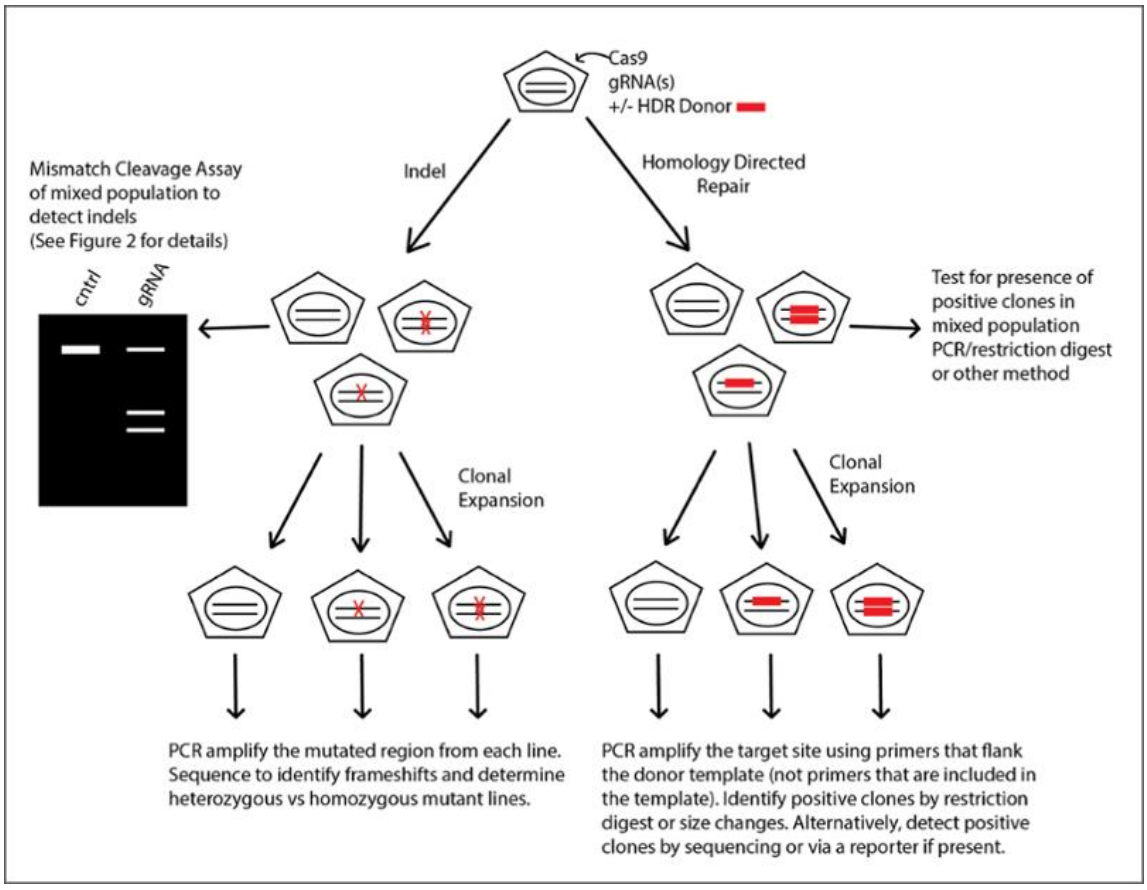

Figure 15. Mismatch cleavage assay and PCR to detect for the presence of positive clones within a mixed population. These techniques are able to increase knockout success by selecting for clones that have both alleles knocked out (Addgene 2015).

Once PCR has been performed and homozygous populations are identified, these can be serial diluted to single colonies which can then be grown up in order to gain a complete homozygous cell line. Western blot can be performed to validate the lack of galectin-1 expression. Studies have knocked out galectin-1 in mice so the large amount of death seen in the transfection stage and the unsuccessful complete knockout of galectin-1 should not be due to galectin-1 being a critical protein and should be able to be completely knocked out (Case et al. 2007; Clark, Weston & Foster 2013; Szebeni et al. 2012).

Although there was no complete knockout, the significant reduction seen in galectin-1 expression means that the cell population could still potentially be used in further studies

to see how galectin-1 expression impacts the ability for *T. vaginalis* to attach to its host. A study by Okumura, Baum & Johnson (2008) demonstrated that a reduction of galectin-1 was able to reduce the parasites ability to bind to the host cells. This study used siRNA to partially reduce the expression of galectin-1 in ectocervical, endocervical and HeLa cell lines (Okumura, Baum & Johnson 2008). As Figure 10 shows, there was quite a significant reduction in galectin-1 expression in the HeLa cell line, which might show interesting results if *T. vaginalis* was interacted with this knockout cell line.

In summary, to further optimize the knockout for future experiments it would be ideal to design sgRNA targets that are more likely to have a higher efficiency and have better on target effects. After transfection but before selection the cells should be sorted through a cell sorter to sort for positive GFP expression, and use a different fluorescent marker instead of GFP when using HeLa cells such as cyan fluorescent protein (CFP). The cells should then be selected for using puromycin and then analysed using PCR to ensure for heterozygous populations. At this point the cells should then be serial diluted and expanded before being analysed using western blot to validate that there is a complete knockout of galectin-1 expression in the cell line. Although this method is more time consuming and costly there is a higher chance of generating a complete knockout in a stable cell line to be used for future experiments.

Chapter 6: Future Directions

6.1 SWATH-MS interaction study

The results discussed above lead to a few potential directions for further studies. Firstly, the SWATH interaction study performed for 30 minutes with 5 x 10^4 parasites / mL (1:5 MOI) should be repeated to obtain independent replicates to strengthen the evidence of what proteins are differentially expressed at this timepoint and to confirm that the proteins were differentially expressed due to interaction of the host cells with the parasite. Further SWATH experiments could also be performed increasing the amount of time that the parasites can attach for to see if the protein profile changes the longer the parasites are attached. The same interaction time (30 minutes) could be used while increasing the number of parasites to see if that changes the expression profile of the mammalian host cells. Finally, these experiments could be performed with different strains of the parasite as different *T. vaginalis* strains have different rates of attachment and cytotoxicity to host cells and thus they would most likely have a unique impact of the protein profile of the host cells (Kucknoor, Mundodi & Alderete 2005).

The proteins that were identified in the study to be differentially expressed could also be studied further to see how *T. vaginalis* manipulates the proteins in the host and what *T. vaginalis* utilizes the protein for. For example, the most interesting differentially expressed proteins are periodic tryptophan protein 1 homolog (Pwp1), serine protease HTRA2 and complement component 1 q subcomponent-binding protein (gC1qR). Studies could be performed to explore Pwp1 upregulation to see if *T. vaginalis* upregulates Pwp1 to promote proliferation of the host cells. Host cells could be interacted with *T. vaginalis* for a longer period of time e.g. a few days to a week to see if the host cells eventually

proliferate or if they all end up dying due to the parasites cytotoxicity to the cells. The experiment could be performed in conjunction with a cell line that has a knockout or knockdown of *PWP1* to see if there is any difference in proliferation. A study exploring serine protease HTRA2 expression could be performed by knocking out HTRA2 and seeing if it effects caspase activity. *T. vaginalis* could be interacted with the knockout cells to see if it effects the parasites survival. Future studies could also look at reducing or increasing iron availability while *T. vaginalis* is interacted to see if this causes *T. vaginalis* to increase its cytotoxic effects in order to cause inflammation and gain nutrients from the environment. Finally, a study could be performed to explore complement component 1 q subcomponent-binding protein (gC1qR). A knockout or knockdown reducing gC1qR expression could be performed and then interacted with *T. vaginalis* to see if this effects the ability for *T. vaginalis* to attach to the host cells.

6.2 Galectin-1 knockout

As discussed above, an incomplete knockout of galectin-1 expression was produced and is most likely due to a mixed cell population. In order to generate a complete knockout, the knockout experiment could be performed again with plasmids that have more efficient sgRNA, the knockout cells could then be analyzed using flow cytometry and sorted by GFP expression or another fluorescent marker that does not clash with HeLa auto fluorescence. The sorted cells could then be selected with puromycin and grown. PCR could then be performed on the cell lines produced by cell sorting to detect for genomic deletions and validate a homozygous deletion. Validated homozygous populations can be serial diluted and then expanded so that the cell population grew from a single cell thus

there should only be homozygous cells in the population once grown. Western blot analysis can then be performed to validate the knockout to validate the complete knockout of protein expression of galectin-1.

Once a complete knockout of galectin-1 is generated, the knockout cell line could be used in a host-parasite interaction study to explore the effects of the knockout on *T. vaginalis* ability to attach to host cells. *T. vaginalis* could be interacted with a control cell line for a period of time before the number of parasites that are attached are counted. This can be repeated on the knockout cell line to then compare whether there was a significant reduction of *T. vaginalis* attachment to the host cells.

Chapter 7: Conclusion

7.1 Conclusion

In conclusion, the aim of this study consisted of two parts; Aim 1: To investigate changes in protein expression in host mammalian cervical epithelial cells following direct interaction of *T. vaginalis* parasites and HeLa cells. Aim 2: A proof of concept study, to successfully knockout host galectin-1 using CRISPR/ Cas9 technology.

This study investigated changes in the protein expression of host mammalian epithelial cells following direct interaction of *T. vaginalis* parasites and HeLa cells. The study identified 22 proteins that were significantly upregulated and downregulated, 8 proteins were identified to be upregulated while 14 were identified to be downregulated. Many of the proteins identified were related to apoptosis, inflammation, downregulating the maturation of proteins as the host cells respond to the cytoxic effects of *T. vaginalis*. The most interesting changes in protein expression were the upregulation of periodic tryptophan protein 1 homolog, serine protease HTRA2 and complement component 1 q subcomponent-binding protein. Further interaction studies could be performed to explore the biological significance of these upregulated proteins potentially finding a therapeutic target to combat *T. vaginalis* infection.

The proof of concept study with the aim to successfully knockout host galectin-1 using CRISPR/Cas9 technology was only able to produce a knockdown of galectin-1 as galectin-1 was still being expressed by the HeLa cells. The knockout could be improved by implementing further techniques that would increase the chances off a successful knockout. This includes cell sorting the transfected cells, validating the knockout with PCR and serial diluting and growing up homozygous clonal cell lines. This knockout

could then be used in a future interaction study to explore the impact of galectin-1 on *T. vaginalis* parasite adhesion.

Combined, this study provides further knowledge of *T. vaginalis* host- parasite interactions and provides insights that could be used in future interaction studies.

Appendix A

SWATH-MS APAF results

https://www.dropbox.com/sh/emtyjebsegwz0ut/AACGmjUZ1FB-K9soa8Ri7Y7Ia?dl=0

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